Identification of a cellulolytic active fungal strain, isolated from a cigarette waste microenvironment, showing preference for growth on cellulose acetate

by

Nicholas George Enslin

Dissertation presented for the degree of
Master of Science (Science)

at
Stellenbosch University
Institute for Plant Biotechnology, Department of Genetics, Faculty of Science

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at are those of the author and are not necessarily to be attributed to the NRF.

Supervisor: DR BIANKE LOEDOLFF
Co-supervisors: DR SHAUN PETERS and PROF JENS KOSSMANN

December 2020

Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 08/10/2020

Abstract

Globally, environmental pollution such as greenhouse gases and microplastics are ubiquitous throughout nature. Long-term negative environmental effects and bioaccumulation of anthropogenic compounds within the food-chain is widely reported. Cigarette filters are the single most littered item with over 4 trillion smoked cigarette filters entering the environment every year. Studies estimate that over 66 % of smokers incorrectly dispose of their cigarette filters culminating to a total annual of 750 million kg of pollution. The fate of an incorrectly discarded cigarette filter causes damage in two major manners. Firstly, a smoked cigarette filter acts as a vector for a myriad of toxic compounds and heavy metals. Secondly, cigarette filters are made of 15 000 or more cellulose acetate fibers linked together by glycerol triacetate. Throughout the degradation process, these toxic compounds and microscopic cellulose acetate fibers leach into the environment. Cigarette filters mostly enter the environment through sewerage and drain water systems that enter into the ocean. Consequently, chemically derived cellulose has been reported covering over 2 billion km^2 of the ocean seabed contributing to the microplastic deep sea sink and marine microplastic epidemic. Research on cigarette filter degradation indicates that after five years, depending on the environment, the total mass loss can range between 50 – 80 %. Estimates suggest that a cigarette filter can remain within the environment for up to ten years. The continual deposition of cigarette filters within the environment highlights the necessity for recycling solutions and effective waste management of cigarette filters.

A cigarette bin could serve as a genetically resourceful environment, where the microbial community partake in a synergistic process for the degradation of cigarette filters. This study centers around a cigarette bin that was theorized to be inhabited by micro-organisms capable of efficiently degrading cigarette filters. The bacterial community within the cigarette bin was previously investigated using 16S small subunit rRNA metagenomic sequencing, as well as a metagenomic library. The aim of this project was to investigate the cigarette bin for cultivatable fungal isolates and select an isolate for *in vitro* enzyme analysis using para-nitrophenyl-linked substrates that mirrors the catabolic pathway of cellulose acetate. Four fungal isolates were cultivated from the cigarette bin and designated I1, I2, I3, and I4. Phylogenetic inference for the four isolates identified as *Mucor circinelloides* f. *circinelloides* (I1, I2, and I3) and *Fusarium proliferatum* (I4). The four isolates were screened *via* multiple functional plate-based screening recipes for the selection of a candidate isolate for *in vitro* enzyme analysis. The

candidate isolate selected was *Fusarium proliferatum* due to the successful screening and observed genetic adaptability towards carboxymethyl cellulose. The *in vitro* enzyme analysis of *Fusarium proliferatum* indicated a β-glucosidase activity of 115.7 nkat/mg of protein towards 4–nitrophenyl–β–D–glucopyranoside and acetyl esterase activity of 157.9 nkat/mg of protein towards 4–nitrophenyl acetate. These preliminary results infer the potential applications of *Fusarium proliferatum* for the remediation of cigarette filter pollution. Valorization of cigarette filters within a fungal-based biorefinery using *Fusarium proliferatum* could generate bioethanol and other high-value products.

Table of Contents

List of Abbreviations

AFRO	African Region
AMRO	American Region
BLAST	Basic Local Alignment Search Tool
BS	Bootstrap
CBS	Central Bureau of Statistics
CLA	Carnation Leaf Agar
CMC	Carboxymethyl Cellulose
CPMAS	Cross-Polarisation Magic-Angle-Spinning
dH_20	Distilled Water
DNA	Deoxyribonucleic Acid
dNTPS	Deoxyribonucleotide Triphosphate
DS	Degree of Substitution
DTT	Dithiothreitol
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetic Acid
EMRO	Eastern Mediterranean Region
EURO	European Region
FCSC	*Fusarium chlamydosporum* species complex
FDA	Food and Drug Administration
FDSC	*Fusarium dimerum* species complex
FFSC	*Fusarium fujikuroi* species complex
FIESC	*Fusarium incarnatum-equiseti* species complex
FOSC	*Fusarium oxysporum* species complex
FSAMSC	*Fusarium sambucinum* species complex
FSSC	*Fusarium solani* species complex
FW	Fresh Weight
GCPSR	Genealogical concordance phylogenetic species recognition concept
gDNA	Genomic Deoxyribonucleic Acid
GTR	General Time Reversible
HEPES	(4-(4-hydroxyethyl)-1-piperazineethanesulfonic) Acid
I1	Isolate 1

I2	Isolate 2
I3	Isolate 3
I4	Isolate 4
ITS	Internal Transcribed Spacer
LB	Luria Broth
MCC	*Mucor circinelloides* complex
MEA	Malt Extract Agar
ML	Maximum Likelihood
MLST	Multilocus Sequence Typing
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
NMR	Nuclear Magnetic Resonance
NMR	Nuclear Magnetic Resonance
NPAHs	Nitropolycyclic aromatic hydrocarbons
NRRL	The Northern Regional Research Laboratory
PAHs	Polycyclic aromatic hydrocarbons
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PMSF	Phenylmethylsulfonyl fluoride
pNP	para-Nitrophenyl
PS	Phylogenetic Species
PVP	Polyvinylpyrrolidone
rRNA	Ribosomal Ribonucleic Acid
SEARO	South-East Asia Region
T92	Tamura 3-Parameter
TBE	Tris-borate-EDTA
tef1	*Translation Elongation Factor 1alpha*
UV	Ultraviolet
WHO	World Health Organization
WPRO	Western Pacific Region

%	Percentage
°C	Degrees Celsius
cm	Centimeter
g	Relative Centrifugal Force
h	Hours
min	Minutes
ml	Milliliter
nkat	Nanokatal
nm	Nanometer
rpm	Revolution Per Minute
s	Seconds
U	Enzyme Activity
v/v	Volume/Volume
w/v	Weight/Volume
µl	Microliter
µM	Micrometer

List of Figures

List of Tables

Chapter 1

Cigarette filters: an environmental pollutant or resourceful genetic toolbox?

Cigarette filters are now considered a greater environmental pollutant than microplastics

A rapid economic development during the 20th century fueled by urbanization and the industrial revolution has resulted in anthropogenic pollution (Alharbi *et al*., 2018; Sexton and Adgate, 1999). An increased rate of pollution (such as greenhouse gasses and microplastics) has given rise to long-term negative environmental effects and bioaccumulation of anthropogenic material within the environment and the food-chain is widely reported (Clarkson, 1995; Rather *et al*., 2017; Yi *et al*., 2008). Recent attention from the scientific community, environmentalists and legislators have raised concerns regarding the negative impact the tobacco industry imposes on both sustainable and responsible economic growth. The World Health Organization (WHO), has spearheaded the implementation of progressive legislation in order to guide Parties on tobacco control (https://www.who.int/tobacco/global_report/en/). Within the last few decades, progressive legislation on tobacco control has been adopted by over 136 countries, protecting five billion people around the world from tobacco products.

Despite the improvement from a policy perspective, the reality has not changed much within the last 40 years. In 2012 alone, 6.25 trillion cigarettes were smoked with an estimated 750 million kg of cigarette filters resulting as litter (Ng *et al*., 2014; Novotny *et al*., 2009). In 2016, the annual number of cigarettes smoked decreased to 5.7 trillion however, this number is predicted to increase again to 9 trillion by 2025 due to the increasing global population (Mackay *et al*., 2006). Tobacco Atlas, an association project of the American Cancer Society and Vital Strategies, indicates that cigarettes smoked per region from 1980 to 2016 suggests future trends for increased numbers of smokers in Asian-, African- and Eastern Mediterranean-regions, while European- and American-regions indicate a steady decline (**Fig. 1**). Additionally, the global per capita of cigarettes smoked indicates a causative effect on global cigarette filter waste with Asian-specific regions having the highest cigarette filter pollution (https://tobaccoatlas.org). The WHO (2017) indicates that up to two-thirds of smokers incorrectly dispose of their cigarette filters, while another study suggests that number is to be above 70 % (Patel *et al*., 2012). As a result, an estimated 4.5 trillion smoked cigarette filters

enter the environment as litter every year. The environmental effects of this continual deposition of cigarette filters within the environment raise serious concerns.

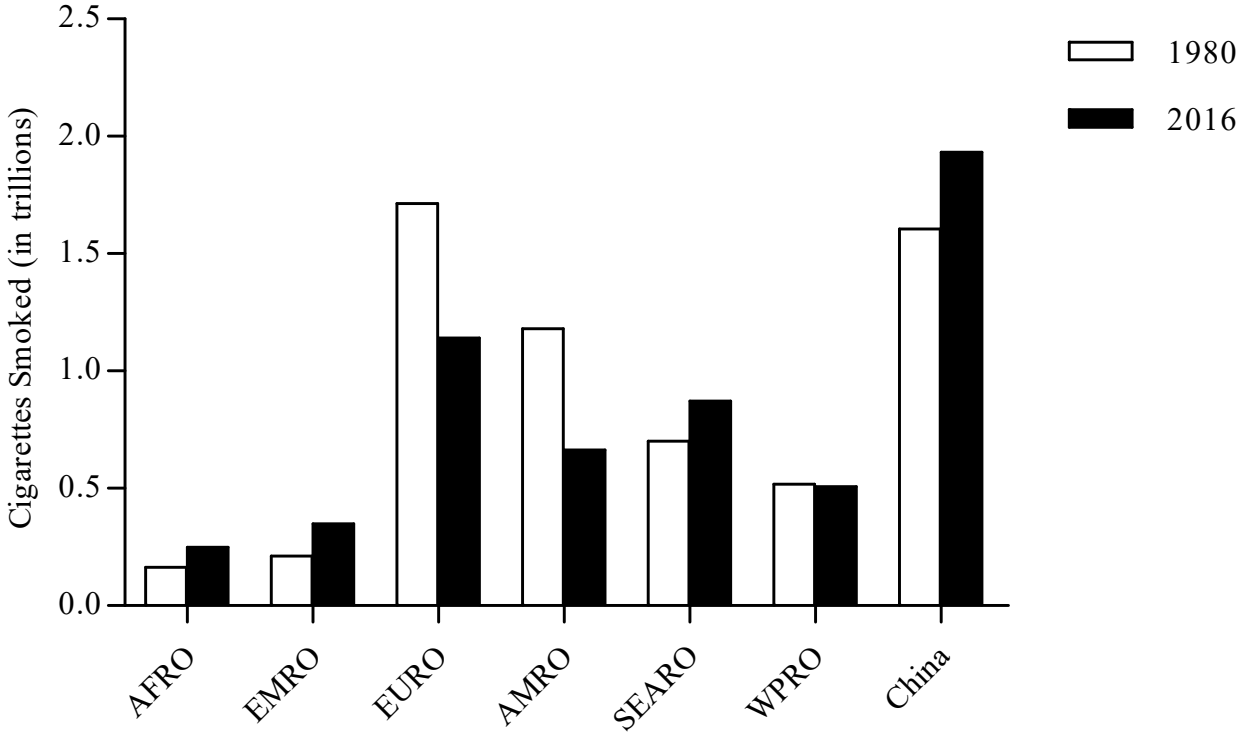

Figure 1: Number of cigarettes smoked in 1980 and 2016 per region in trillions. AFRO: African Region; EMRO: Eastern Mediterranean Region; EURO: European Region; AMRO: American Region; SEARO: South-East Asia Region; WPRO: Western Pacific Region (excluding China).

Environmental reports indicate that cigarette filters are a hazardous anthropogenic material within the environment, culminating in one of the biggest risk factors to both terrestrial and marine ecosystems (Araújo and Costa, 2019a, 2019b; Booth *et al*., 2015; El Hadri *et al*., 2020; Green *et al*., 2019, 2020; Kataržytė *et al*., 2020; Moriwaki *et al*., 2009; Micevska *et al*., 2006; Novotny and Slaughter, 2014; Slaughter *et al*., 2011; Torkashvand *et al*., 2020; Wright *et al*., 2015). The Ocean Conservancy has annually sponsored beach clean-ups since 1986, with cigarette filters topping the list consecutively every year; a total of over 60 million cigarette filters collected (Ocean Conservancy, 2018). The extent of cigarette filter pollution is highlighted in sampling studies indicating cigarette filters are the most littered item contributing to 6 – 14 % of total litter recovered from beaches in Europe (Addamo *et al*., 2017). More recently, cigarette filters were determined to contribute up to 41 % of total items from Lithuanian beaches and up to 85 % of total items from German beaches (Kataržytė *et al*., 2020). This correlates to 0.54 cigarette filters/m^2 in Lithuania and 29 cigarette filters/m^2 in Germany. Other studies have reported 13.3 cigarette filters/m^2 (Thailand) and 38 cigarette filters/m^2

(Persian Gulf) from beaches within these locations (Dobaradaran *et al*., 2018; Kungskulniti *et al*., 2018). Such sampling studies focused on cigarette filter pollution indicate to the severity of cigarette filters, the single most littered item.

Decomposing cigarette filters result in the leaching of several toxic compounds into the environment

Cellulose acetate forms part of a collective material known as rayon; a non-plastic, semi-synthetic material derived from chemical processing of cellulose. Rayon contributes 6.2 % of global fiber production, an annual total of 6 700 million kg, and is used in numerous commercial sectors such as hygiene products, clothing manufacturing and cigarette filter production (Suaria *et al*., 2020). Mostly, rayon enters the environment through sewerage and drain water systems that connect to the ocean. A key property of marine debris is the density of the material with respect to seawater. Cigarette filters have a specific gravity of 1.22-1.24, resulting in a rapid negative buoyancy effect (Andrady, 2011; Lobelle and Cunliffe, 2011). The previously unaccounted extent of global microplastics and microfibers has been resolved by identifying the ubiquitous prevalence of microplastic fibres on the ocean seabed, indicating four times more microplastics within deep sea samples compared to that of water surface samples (Gago *et al*., 2018; Cozar *et al*., 2014; Woodall *et al*., 2014). The exact percentage that cigarette filters contribute to the marine microplastic crisis is unknown, however, the sheer number of cigarette filters annually resulting as litter is noteworthy and concerning (Andrady, 2011).

Consequently, the major environmental concern of a cigarette filter are the cellulose acetate fibres that constitute the cigarette filter. A cigarette filter is made of approximately 15 000 or more cellulose acetate fibres that are linked together by a plasticizer known as glycerol triacetate (Hon, 1997; Novotny and Slaughter, 2014). As the cigarette filter begins to degrade, these microscopic cellulose acetate fibres fragment and leach into the environment. Consequently, rayon is widely reported in marine environments covering 2 billion km^2 of ocean seabed, forming part of the microplastic deep sea sink and marine microplastic epidemic (Woodall *et al*., 2014). Additionally, rayon has been identified in the gastrointestinal tract of fish (57.8 % of synthetic particles identified were rayon derived) and ice cores (54 % of synthetic particles identified were rayon derived) which further illustrates the ubiquitous nature thereof (Lusher *et al*., 2013; Obbard *et al*., 2014).

Compounded to cigarette filter pollution, during the biodegradation period, the smoked cigarette filter acts as a vector for a myriad of toxic compounds and heavy metals retained within the filter (**Table 4**; Supplementary data)**.** Cigarette filters can contain over 4 000 chemicals consisting of various alcohols, alkaloids, aromatic amines, carbonyls, hydrocarbons, insecticides, metals, nitrosamines, NPAHs, PAHs, phenols, phthalates, pyrazines, pyrroles, terpenes and terpenoids (Hoffmann and Hoffmann, 1997; Novotny *et al*., 2009; Poppendieck, *et al*., 2016; Slaughter *et al*., 2011). Of this extensive list of chemicals within cigarette filters and tobacco, many are noted as harmful or potentially harmful constituents by the US Food and Drug Administration (FDA). These toxic compounds found within cigarette filters leach into the environment, as many, such as nicotine, are soluble. Recent studies provide some quantification of the negative impact cigarette filters impose on marine and terrestrial wildlife. Cigarette filter pollution has been shown to effect a range of organisms with respect to adverse growth, changes in physiology, behavioural variations, genotoxicity, and cytotoxicity (Booth *et al*., 2015; Di Giacomo *et al*., 2015; Green *et al*., 2019; Micevska *et al*., 2006; Slaughter *et al*., 2011; Wright *et al*., 2015).

Commercially available cigarettes are made up of various components and contain several toxic compounds (**Fig. 2**). After the smouldering phase of a cigarette, remnant tobacco and the cigarette filter remain. Once discarded, the fate of a cigarette filter is subject to two main forms of degradation: photodegradation and biodegradation (Puls *et al*., 2010). Photodegradation is an important route for the degradation of cigarette filters within the environment (Puls *et al*., 2010). However, the rate of degradation is not influenced significantly through photodegradation alone. Cellulose acetate has an absorption wavelength of ~260 nm, which is less than the 300 nm threshold wavelength of sunlight entering the earth's atmosphere (Hosono *et al*., 2007; Jortner *et al*., 1959). This suggests that the formation of free radicals initiated through absorption of sunlight does not occur from cellulose acetate. Rather, secondary mechanisms of photodegradation occur when contaminates enter the system capable of absorbing sunlight and subsequently producing free radicals eliciting photocatalytic oxidation or photosensitized degradation of cellulose acetate (Puls *et al*., 2010). Although now considered biodegradable, early reports on cellulose acetate falsely labelled the material as non-biodegradable (Potts *et al*., 1972). This was largely due to drawing comparisons to cellulose degradation without considering additional enzymatic mechanisms required. Currently, we understand the rate limiting step of cellulose acetate degradation to be deacetylation, the key mechanism for the initiation of cellulose acetate degradation. (Ho *et al*.,

1983; Samios *et al*., 1997). Nevertheless, using carbon-13 cross-polarization magic-angle-spinning nuclear magnetic resonance (^{13}C CPMAS NMR), a study investigated the degradation of cigarette filters under various conditions and revealed that after two years 20 – 30 % of the filters initial mass was lost (Bonanomi *et al*., 2015). While after five years, depending on the environment the cigarette filters were incubated, 50 – 80 % of the cigarette filters initial mass was lost (Bonanomi *et al*., 2020). No peer-reviewed work on long-term cigarette filter decomposition is available however, estimates suggest that cigarette filters can remain within an environment for up to ten years.

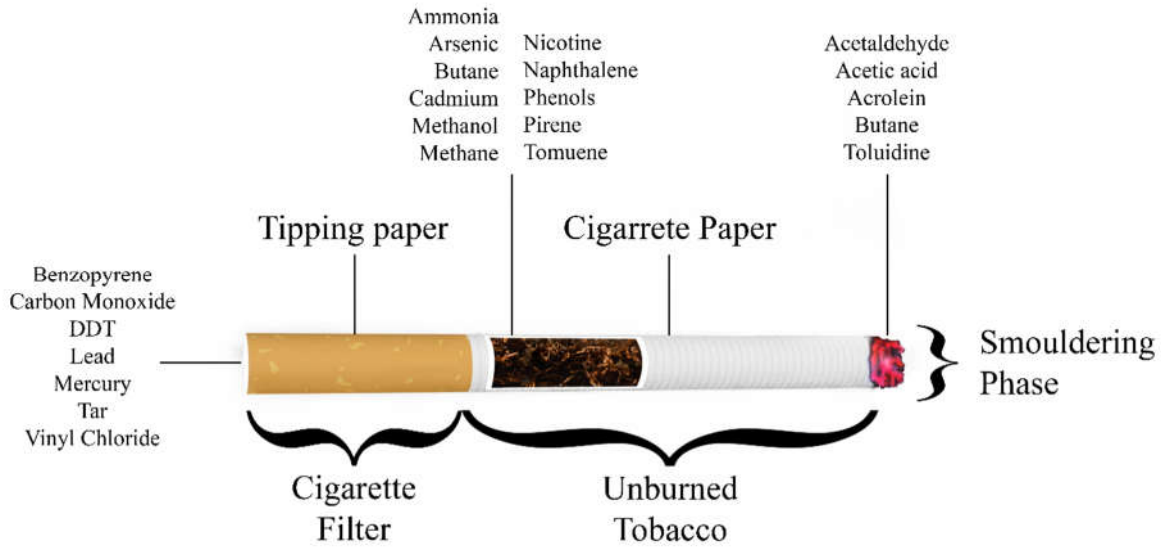

Figure 2: A commercially available cigarette listing the main components and several toxic compounds. After the smouldering phase, remnant tobacco and the cigarette filter remain. The major chemicals noted as harmful within the different components of a cigarette are listed (image adapted from Marinello *et al*., 2020).

The 'microbiome' of a cigarette bin: potential for natural decomposers

The degradation of anthropogenic material requires a complex interplay of geochemical, physical, and biological factors within a polluted environment (Alharbi *et al*., 2018). Consequently, the structure and dynamics of micro-organisms are largely responsible for the *in situ* degradation of anthropogenic material (Atlas, 1981; Rhee *et al*., 2004). From an ecological perspective, cigarette filter pollution imposes a serious threat to both marine and terrestrial wildlife. However, from a genomic perspective, a cigarette bin could serve as a resourceful environment where the microbial and fungal community partake in a synergistic

process for the degradation of cigarette filters (Puls *et al*., 2010). The community of micro-organisms within a cigarette bin would herald enzymes capable of degrading cigarette filters, including the toxic chemicals and heavy metals associated with the environment (Mittal *et al*., 2019; Reinthaler *et al*., 2003). This microbiome within the cigarette bin would therefore also encompass the total genomic DNA for the establishment of a complete degradative pathway for cigarette filters. In this regard, the microbial ecology of a cigarette bin could potentially lead to the identification of novel species/enzymes while determining potential remediation solutions for cigarette filter pollution.

Understanding how degradative pathways are established from novel substrates centres at the crux of evolutionary biology. Research suggests that novel enzyme activities evolve from changes within promiscuous ancestral enzymes (Guzmán *et al*., 2019). Promiscuous ancestral enzymes permit early mutational events for novel capabilities and phenotypic improvements (Khersonsky and Tawfik, 2010). Evolutionary changes within these regions of DNA allow for the generation of novel metabolic pathways from non-native substrates (Copley, 2000; Huang *et al*., 2012; Jensen, 1976; Schmidt *et al*., 2003). Mutational events and the genetic adaptability of an organism during the growth on non-native substrates highlights the complexity of evolutionary dynamics and physiological mechanisms (Khersonsky and Tawfik, 2010). Expanding on this concept, the comparison between gradual genetic improvements and early mutations must be distinguished. Gradual genetic improvements result in improved enzyme kinetics for substrate specificity or, adapting expression through regulatory elements. Early beneficial mutations endow an organism with novel enzymatic capabilities enabling the proliferation within a new ecological niche (Barrick and Lenski, 2013; Wagner, 2011).

Although studies do expand on the microbial ecology of tobacco leaves, cured tobacco, unsmoked cigarettes during storage, and the human microbiome of smokers; cigarette filter waste is under-reported (Chopyk *et al*., 2017; Huang *et al*., 2010; Pauly *et al*., 2007; Sapkota *et al*., 2010; Zhou *et al*., 2020). Species such as *Bacillus, Kurthia* and *Mycobacterium* were identified in smoked cigarette filters; however, these studies are limited due to their inherent experimental design (Eaton *et al*., 1995; Rooney *et al*., 2005). Recent studies include, a five-year decomposition experiment focused on both the degradation and microbial ecology of smoked cigarette filters incubated in different environments (Bonanomi *et al*., 2015, 2020). The microbial ecology was investigated through high-throughput sequencing of bacterial and

fungal rRNA markers (16s rRNA and BITS2F/B58S3, respectively). Linking both the degradation and microbial ecology of smoked cigarette filters revealed that the unique changes in the microbiota of cigarette filters expedited the degradation process (Bonanomi *et al*., 2015, 2020). Although this study is based on a culture-independent approach, it serves as the cornerstone of the microbial ecology of smoked cigarette filters.

Fungi: the decomposers of this world and their biotechnological applications

Fungi serve an important role on earth as decomposers. As heterotrophs, fungi have no photosynthetic complexes and obtain the necessary energy, carbon, and nutrients through the degradation of biomass constituents and other already existing molecules (Dix and Webster, 1995). Their responsibility as carbon and nitrogen cyclers is mostly characterised by leaf litter and their ability to recycle the previous season of fallen leaves (Rai and Srivastava, 1983). This extends to their role in the soil food web, with fungi being the primary candidates in degrading complex molecules such as lignin and cellulose. However, before smaller molecules such as sugars, amino acids, and organic acids can be transported and utilized within the cell, these complex molecules within the environment need to be broken down. Fungi achieve this by secreting a suite of enzymes that initiate the biocatalytic degradation of specific complex molecules (Gopinath *et al*., 2005; Hankin and Anagonostakis, 1975). Fungi occupy a wide range of habitats with numerous survival mechanism and novel metabolic pathways. As a result, fungi have historically, and continue to serve as one of the major candidates for identifying novel enzymes and metabolites of industrial and pharmaceutical relevance (Hyde *et al*., 2019).

Cigarette filter pollution could serve as a carbon source for fungal whole-cell growth and metabolism. Fungi can be cultivated with minimal effort on cheap and nutrient-poor substrates, providing a sustainable and scalable business model. The general trend of harvesting and isolating fungi from unique environments for specific biotechnological applications enters a massively scalable business model for a variety of industry-consumer products (**Fig. 3**; Hyde *et al*., 2019). This general trend is outlined by initially scanning a specific environment through molecular sequencing of DNA barcodes, followed by literature search of identified species, gene editing (if viable in the consumer market), storage of isolates for reproducibility, and finally applying the research in industry. To date, consumer markets from fungal-based biorefineries have benefited from the production of novel compounds, feedstock, bio-

fertilizers, bio-controlling agents, industrially relevant enzymes, composite materials, cosmetics, medicines, post-harvest control, and remediation of pollutants (**Fig. 3**; Hyde *et al*., 2019). In this regard, a fungal-based biorefinery for the remediation of cigarette filter pollution could potentially feed into a multitude of high-value products such as glucose or bioethanol. This is important for the valorisation of cigarette filters as recycling solutions need financial incentive. Economic estimates for collecting cigarette filters in San Francisco was reported to cost between $0.5 – $6 million annually (Marah and Novotny, 2011; Rath *et al*., 2012). To decrease cigarette filter pollution (a generally under-funded venture), economic bootstrapping through the extraction of high-value products from a fungal-based biorefinery provides an alternative for the valorisation of cigarette filter pollution.

Within the realm of industrial enzymes and pharmaceuticals, there is great potential for the valorisation of cigarette filters within a fungal based biorefinery. The global market for industrial enzymes is valued at over $6 billion dollars, of which ~50 % are fungal derived (Cabanne and Doneche, 2002). These industries include, but are not limited to, paper and textile production, biofuel, food, and agriculture (Erickson *et al.*, 2012; Kuhad *et al*., 2011; Singh *et al*., 2016). Industrial enzymes offer many advantages over traditional chemical processes regarding sustainability and efficiency. Within the pharmaceutical industry, there are many fungal derived antibiotics, cholesterol-lowering agents and immunomodulatory agents such as pravastatin (~$3.6 billion/year), cyclosporine (~$1.4 billion/year), amoxicillin (~$11.7 billion/year), fingolimod (~$1 billion/year) and most famously, penicillin (Raja *et al*., 2017). In this regard, the general trend for harvesting and isolating fungi for the remediation of cigarette filters can feed into specific industry-consumer markets that de-risk the overall process of reducing cigarette filter pollution.

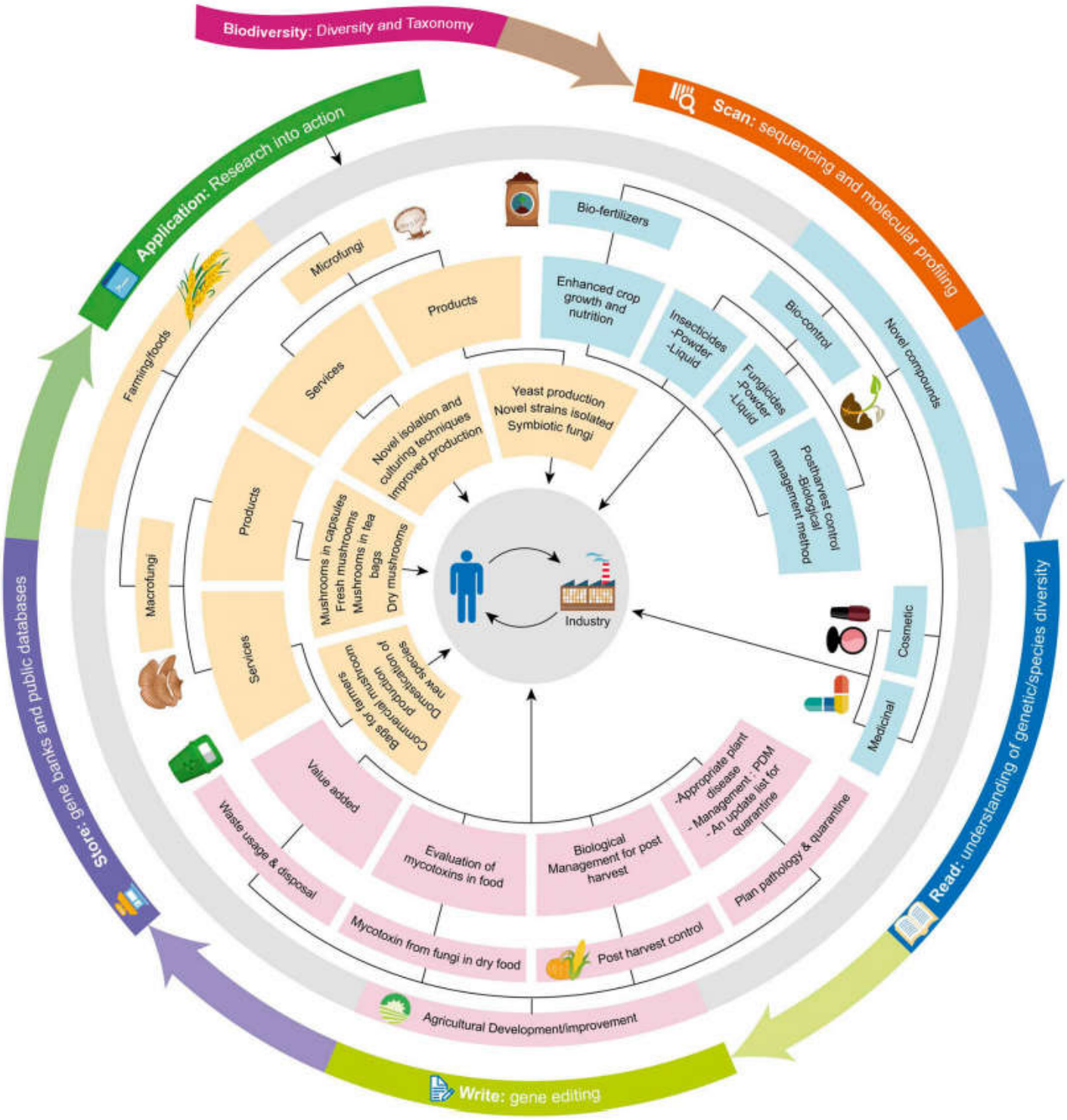

Figure 3: An evaluation of the biotechnological applications of fungi and potential consumer markets. The process begins with investigating the fungal ecology of specific environments for novel fungi. Harvesting fungal isolates expands international culture collections and current phylogenetic concepts. The fungal isolates are then characterized for industrial applications. Using fungi for industrial applications may be applied to farming/food, agricultural development, and production of novel compounds (image from Hyde *et al*., 2019).

Toward identifying fungal species within polluted environments

The discovery and classification of new fungal species is a challenging task for mycologists. The seemingly continuous identification of novel fungi within the advent of molecular biology revealed an unexpected fungal biodiversity (Hawksworth, 2004; Hibbetts *et al*., 2011). Culture-dependent approaches have described over 100 000 fungal species to date, which has then been challenged by culture-independent approaches and molecular techniques. Several novel taxa, divisions, classes, orders, and families have been established in the last few decades. Molecular sequencing and phylogenetics revealed many morphological similar taxa were in fact distinct lineages, and many species were in fact within a species complex (Dai *et al*., 2015; Wu *et al*., 2019). Additionally, molecular sequencing for phylogenetic inference resolved cryptic speciation; two or more different fungal species with similar morphological and physiological traits (Wu *et al*., 2019).

The general trend within the last nine years has shown a precedent towards molecular identification; a direct result of affordable sequencing reactions and universal fungal DNA barcodes (Raja *et al*., 2017). Simply, DNA barcodes are specific regions of DNA that are sequenced and compared to genetic databases for rapid species identification (https://ibol.org/about/dna-barcoding/). In 2011, a multinational consortium of mycologists considered six regions of DNA for a universal DNA barcode for fungi (Schoch *et al*., 2012). Out of the six proposed barcodes, the Internal Transcribed Spacer (ITS) region was approved, resulting in the International Barcode of Life to designate the ITS region as the official barcode for fungal species identification. The ITS region contains two variable non-coding regions placed either side of the highly conserved rDNA 5.8S small subunit (Gardes and Bruns, 1993; Schoch *et al*., 2012). This divergent region of DNA is capable of hyper variation and is the fastest evolving rRNA cistron. The ITS region encapsulates the essence of a DNA barcode in that the interspecific variation exceeds the infraspecific variation enabling species-level identification (Bruns *et al*., 1991; Raja *et al*., 2017). As a DNA barcode for fungal species identification, the ITS region has been determined to have the highest probability for successful species-level identification (Schoch *et al*., 2012). Over 170 000 full length ITS sequences have been deposited in GenBank; of which 56 % are annotated with a Latin binominal. This represents over 15 000 fungal species, 2 500 genera published in over 11 500 scientific studies in 500 peer-reviewed journals (Schoch *et al*., 2012). This wealth of sequence information enables mycologists a stable classification system for fungal species identification. Remaining

ITS sequences that have not been annotated a Latin binominal often are the result of environmental DNA sequencing and other culture-independent approaches (Buée *et al*., 2009; O'Brien *et al*., 2005). Consequently, using the GenBank NCBI BLAST search for fungal species identification can be misleading and therefore should be avoided or, proceeded with caution (Prakash *et al*., 2017; Wu *et al*., 2019). Mycologists expressed concerns of using this common route for fungal species identification, indicating that 27 % of GenBank fungal ITS sequences have insufficient taxonomic data, with 20 % of all fungal sequences in GenBank incorrectly annotated (Bridge *et al*., 2003; Kõljalg *et al*., 2013; Nilsson *et al*., 2008; Raja *et al*., 2017; Vilgalys, 2003). For phylogenetic analyses, engaging fungal databases for verified nucleic acid sequences is crucial, with a general pipeline as follows: (i) accessing the database, (ii) strategizing a particular DNA barcode search, (iii) executing the search, iv) retrieving the data (Prakash *et al*., 2017). Reliable and frequented databases within mycology include: BOLD, CBS-KNAW, AspGD, *Fusarium* MLST, ISHAM-ITS, MycoBank, Mycology Online, Doctor Fungus, FungiDB, and UNITE. Accessing databases provides reliable taxonomy, nomenclature, identification, and genotyping of fungal isolates. Ultimately, no database is perfect, as all databases have a proclivity towards obsoletion, and thus require consistent curation and engagement from researchers.

Although the ITS region as a DNA barcode is well suited for many fungal organisms, it can be uninformative or misleading for species within the genus *Penicillium, Trichoderma, Fusarium, Cladosporium and Aspergillus*. For successful implementation of a DNA barcode, the following criterion must be met: i) must be applicable across the entire genus, ii) must be informative at a species-level and iii) orthologous across the genus (O'Donnell *et al*., 2015). An alternative to the ITS region, protein-coding genes are suited as a DNA barcode with improved phylogenetic resolution within the genus of certain fungi (Schoch *et al*., 2009). Protein-coding genes are capable of hyper variation, with high levels of homology and convergence and the added benefit of preferential sequence alignment due to codon restraints (Berbee and Taylor, 2001; Raja *et al*., 2017). Well accepted protein-coding DNA barcodes within the fungal community include the *translation elongation factor 1-alpha* (*tef1*), the subunits of *RNA polymerase* (*RPB1* and *RPB2*), and *beta-tubulin* (*tub2*; Glass and Donaldson, 1995; Hibbett *et al*., 2007; James *et al*., 2006; Liu and Hall, 2004; Matheny *et al*., 2002; O'Donnell and Cigelnik, 1997; Rehner and Buckley, 2005; Schoch *et al*., 2009). The inclusion of these phylogenetic markers within fungal systematics has enabled a stable classification of

fungi and broadened the current understanding of fungal taxonomy and evolution (Hibbett *et al*., 2007).

Research on the microbial ecology of polluted environments has highlighted the interesting structure and dynamics of bacterial and fungal communities (Fabiano *et al*., 1994; Ford, 1994; Kandeler *et al*., 2000; Kraemer *et al*., 2019; Labbate *et al*., 2016; Müller *et al*., 2001; Röling *et al*., 2001; Ventorino *et al*., 2018). Using DNA barcodes for the identification of novel fungi within polluted environments expands potential industrial applications, while providing reproducibility, standardisation, and access to broader species information. Interlinking the pollutant type and rate of degradation to the fungal ecology provides a better understanding of *in situ* biochemical mechanisms involved. Understanding the biological factors responsible for the degradation of cigarette filters by fungi could provide efficient solutions for the remediation of cigarette filter pollution (discussed further in **Chapter 2**).

Aims and objectives

This research project forms part of a larger initiative focused on identifying the microbial community associated to a cigarette bin located at the Institute of Plant Biotechnology, Stellenbosch University. The wastebin was utilized for discarded smoked cigarette filters and associated to biological detritus for fifteen years. We observed that the smoked cigarette filters at the bottom of the wastebin were rapidly degraded within a year. Subsequently, the cigarette bin was nurtured as a scientific experiment for the characterization of the microbial community and associated open reading frames (ORFs). The bacterial community within the cigarette bin was previously investigated using 16S small subunit rRNA metagenomic sequencing. Additionally, a fosmid metagenomic library was generated from the microbial community inhabiting the cigarette bin. The metagenomic library was functionally screened for cellulolytic and acetyl esterase enzyme activity (Pieters, 2018 available at http://hdl.handle.net/10019.1/105187).

The aim of this project was to further investigate the cigarette bin for cultivatable fungal isolates. This chapter includes the identification and characterization of four fungal isolates harvested from the cigarette bin. Fungal spores were harvested from the cigarette bin and cultivated under standard laboratory conditions. The fungal isolates were morphologically characterized *via* light microscopy. gDNA was extracted from the fungal isolates and specific DNA barcodes were cloned and sequenced. The sequenced DNA barcodes were analysed *via* phylogenetic inference and lead to the successful identification of the cultivatable fungi harvested from the cigarette bin.

1.1 Materials and methods

1.2.1 Fungal cultivation and morphological analyses

Fungal spores were harvested from a cellulose acetate-rich environment (cigarette bin) at the Institute of Plant Biotechnology, South Africa (33.9328° S, 18.8644° E). Material harvested from the cigarette bin was transferred to flasks containing 50 ml dH_2O and incubated in the dark (25-30 °C). Serial dilutions were plated on malt extract agar (Sigma Aldrich, South Africa; 72 h, 25 °C in the dark; MEA; 4 % potato dextrose agar (w/v), 2 % malt extract (w/v), 0.6 % peptone (w/v)). Subsequently, four fungal axenic cultures were cultivated, and fungal spores were suspended in dH_2O and 0.1 % Tween 80 (v/v). The harvested spores were maintained in dH_2O and 0.1 % Tween 80 (v/v) for short term storage (4 °C) or in 10 % glycerol (v/v) for long term cryogenic storage (-80 °C).

The gross morphology of the four fungal isolates when grown on MEA (10 days, 25 °C in the dark) were photographed. Both the top view and bottom view of the petri dish was photographed, for a comprehensive analysis of the gross morphology. The microscopic morphological characteristics of the four fungal isolates were assessed by cultivating spores on MEA and carnation leaf agar (72 h, 25 °C in the dark; CLA; 0.5 % agar (w/v), sterile carnation leaves). Microscopic morphology was examined *via* light microscopy on an Ax10 Scope.A1 microscope fitted with an Axiocam 305 Color camera (Zeiss, Germany). Microscope slides were prepared by mounting fungal tissue stained with 10 µl cotton lactophenol blue (0.0625 % cotton blue (w/v), 2 5 % phenol (w/v), 50 % glycerol (w/v), 25 % lactic acid (v/v)). The focus was on hyphae, microconidia, macroconidia, chlamydospores and spores.

1.2.2 DNA isolation, DNA amplification and species identification

Total genomic DNA (gDNA) was extracted from fungal mycelia (100 mg FW; fresh weight), cultivated on MEA (72 h, 25 °C in the dark), using the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research, Inqaba Biotech, South Africa), according to the manufacturer's instructions. As recommended by the manufacturer, 0.5 % β-mercaptoethanol (v/v) was added to the DNA binding buffer for optimal nucleic acid extraction. Species identification was achieved through sequence typing of the ITS region for three of the fungal isolates using the ITS5 and ITS4 primer pair (ITS; **Table 2**; White *et al*., 1990), and partial sequence typing of

the *translation elongation factor 1α* for one of the fungal isolates using the EF1 and EF2 primer pair (*tef1*; **Table 2**; O'Donnell *et al*., 1998).

PCR reactions were performed in a T100™ Thermal Cycler (Bio-Rad, South Africa) with the following reagents: 5x Q5 reaction buffer, 10 µM primers, 10 µM dNTPs mix, and 0.02 U/µl Q5 High-Fidelity DNA Polymerase, in a total reaction volume of 50 µl (Q5 DNA polymerase, New England Biolabs, Inqaba Biotech, South Africa). PCR thermocycling conditions for the ITS region were as follows: an initial denaturation (98 °C, 30 s), 25 cycles (98 °C, 10 s; 61 °C, 30 s; 72 °C, 30 s) and a final extension (72 °C, 2 min). The PCR thermocycling conditions for *tef1* were as follows: an initial denaturation (98 °C, 30 s), 25 cycles (98 °C, 10 s; 57 °C, 30 s; 72 °C, 30 s) and a final extension (72 °C, 2 min). PCR products were visualized under UV gel electrophoresis (80 V) on 1 % TBE (tris/Borate/EDTA; 10.8 % tris (w/v), 5.5 % boric acid (w/v), 4 % EDTA pH 8.0 (v/v)) agarose (w/v) stained with ethidium bromide. The 1 kb DNA ladder (Promega, Anatech, South Africa) was used to discriminate the sizes of the amplicons. Subsequently, the PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The purified PCR products were then cloned into pMiniT 2.0 and subsequently transformed into NEB 10-beta Competent *E.coli* (**Table 1**; New England Biolabs). Plasmid extractions were conducted using the Wizard® Plus SV Miniprep DNA Purification System (Promega). Plasmid amplicons were sequenced in both directions using the plasmid cloning analysis primers (**Table 2**; New England Biolabs).

1.2.3 Phylogenetic inference for the fungal isolates harvested from a cellulose-acetate rich environment

Consensus sequences were determined and assembled on the CLC Genomics Workbench (v12.0.3, Qiagen, Whitehead Scientific, South Africa) and compared to representative ITS and *tef1* sequences from previous studies (O'Donnell *et al*. 1998; O'Donnell *et al*., 2010; Groenewald et al., 2019). Nucleic acid sequences for both ITS and *tef1* sequence datasets were retrieved from the CBS-KNAW Fungal Biodiversity Centre's Fusarium MLST database (https://fusarium.mycobank.org/) and the NCBI database (https://www.ncbi.nlm.nih.gov/). The sequences were used in the construction of two phylogenetic trees, using the program MEGA X and the algorithm ClustalW with manual corrections where necessary (Tamura et al. 2013). Phylogenetic inference in this study was based on Maximum Likelihood (ML). Clade stability was tested with a bootstrap analysis of 10 000 replicates using a 70 % bootstrap (BS)

criterion. The outgroup *Parasitella parasitica* (CBS 208.28) was used in the construction of the ITS *Mucor* phylogenetic tree, while *Stachybotrys chartarum* (CBS 129.13) was used in the construction of the *tef1 Fusarium* phylogenetic tree. The *in-silico* analyses were completed using a high-performance computer (HPC), housed at the Institute of Plant Biotechnology. The basic hardware specifications include 35-core processing power (x2 Xeon 17 core processors with clock speed of 3 gigahertz (GHz) per core), 64 gigabytes (gB) of random-access memory (RAM) – enabling rapid analyses of high throughput data sets. The topology of the two phylogenetic trees were developed in FigTree (version 1.4.4; Rambaut, 2009), with colour correction edited in Adobe® Photoshop CC (version 14.0).

Table 1: Strain of *E. coli* and vector used in this study.

Name	Genotype	Reference/source
NEB® 10-beta Competent *E. coli*	Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- ϕ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (StrR) rph spoT1 Δ(mrr-hsdRMS-mcrBC)	NEB, Inqaba Biotechnical Industries
pMiniT 2.0	Cloning of partial genes for multilocus sequence typing	NEB, Inqaba Biotechnical Industries

Table 2: Primer sequences used in this study.

Designation	Primer Sequence 5' → 3'	Reference
	Internal transcribed spacer (ITS)	
ITS4	TCCTCCGCTTATTGATATGC	White *et al*., 1990
ITS5	GGAAGTAAAAGTCGTAACAAGG	White *et al*., 1990
	Translation elongation factor 1α (tef1)	
EF1	ATGGGTAAGGARGACAAGAC	O'Donnell *et al*., 1998
EF2	GGARGTACCAGTSATCATG	O'Donnell *et al*., 1998
	NEB® PCR Cloning Kit	
Cloning Analysis Forward	ACCTGCCAACCAAAGCGAGAAC	NEB, Inqaba Biotechnical Industries
Cloning Analysis Reverse	TCAGGGTTATTGTCTCATGAGCG	NEB, Inqaba Biotechnical Industries

1.3 Results

1.3.1 Morphological characterization of fungi harvested from the cigarette bin

A culture-dependent approach was conducted in this study which led to the cultivation of four fungal isolates under standard laboratory conditions and were designated I1, I2, I3 and I4 (**Fig. 4**). The morphological features of I1, I2, and I3, are characterized by sporangia formed on repeatedly sympodial branched sporangiophores with non-truncated columellae (**Fig. 5**). The size of sporangia is less than 100 µm in diameter with the size of sporangiospores less than 13 µm in diameter and less than 10 µm in length (**Fig. 5**). For I4, the use of CLA media was required for induction of macroconidia. Morphological features of I4 are characterized by slender sickle macroconidia less than 2 µm in length, false heads containing microconidiophores (8 – 16), a white villous gross morphology and a light purple hue when grown on MEA (**Fig. 4**; **Fig. 5**; Sun *et al*., 2018). Similar morphological features found in literature loosely placed I1, I2, and I3 in the *M. circinelloides* complex (MCC), while I4 was loosely placed in the *Fusarium fujikuroi* species complex (FFSC; Wagner *et al*., 2019; Walther *et al*., 2013; Sun *et al*., 2018). Both the genus *Mucor* and *Fusarium* contain cryptic species, limiting the utility of morphological characterization for species-level identification.

1.3.2 DNA amplification and molecular sequencing

PCR amplification of the ITS region for I1, I2, and I3, using the ITS5 and ITS4 primer pair (**Table 2**), was confirmed, representing a ~650 bp amplicon (**Fig. 6**). Although the ITS region from I4 was amplified and sequenced it immediately became evident that I4 required a protein—coding DNA barcode for successful species identification. The ITS region is uninformative for the genus *Fusarium* and was therefore discarded from the phylogenetic analysis. PCR amplification of the *tef1* for I4, using the primer pair EF1 and EF2 (**Table 2**), was confirmed, representing a ~700 bp amplicon (**Fig. 6**).

In order to conserve genetic information on the 5' and 3' end, the PCR amplicons were cloned into the into pMiniT 2.0 and sequenced using the cloning analysis primers (**Table 2**) *via* capillary sequencing. The sequencing of the ITS region for I1, I2 and I3 indicated successful amplification of the ITS1, 5.8S and ITS2 region. I1 indicated a 621 bp ITS region sharing 98 % (612/621) and 98 % (610/621) sequence identity with I2 and I3, respectively. Both I2 and I3 indicated a 620 bp ITS region sharing 99 % (618/620) sequence identity. Sequencing of *tef1*

for I4 indicated a 594 bp amplicon, with successful amplification of the 5' end of *tef1* with primers binding within conserved exons and expanding between three introns and two exons.

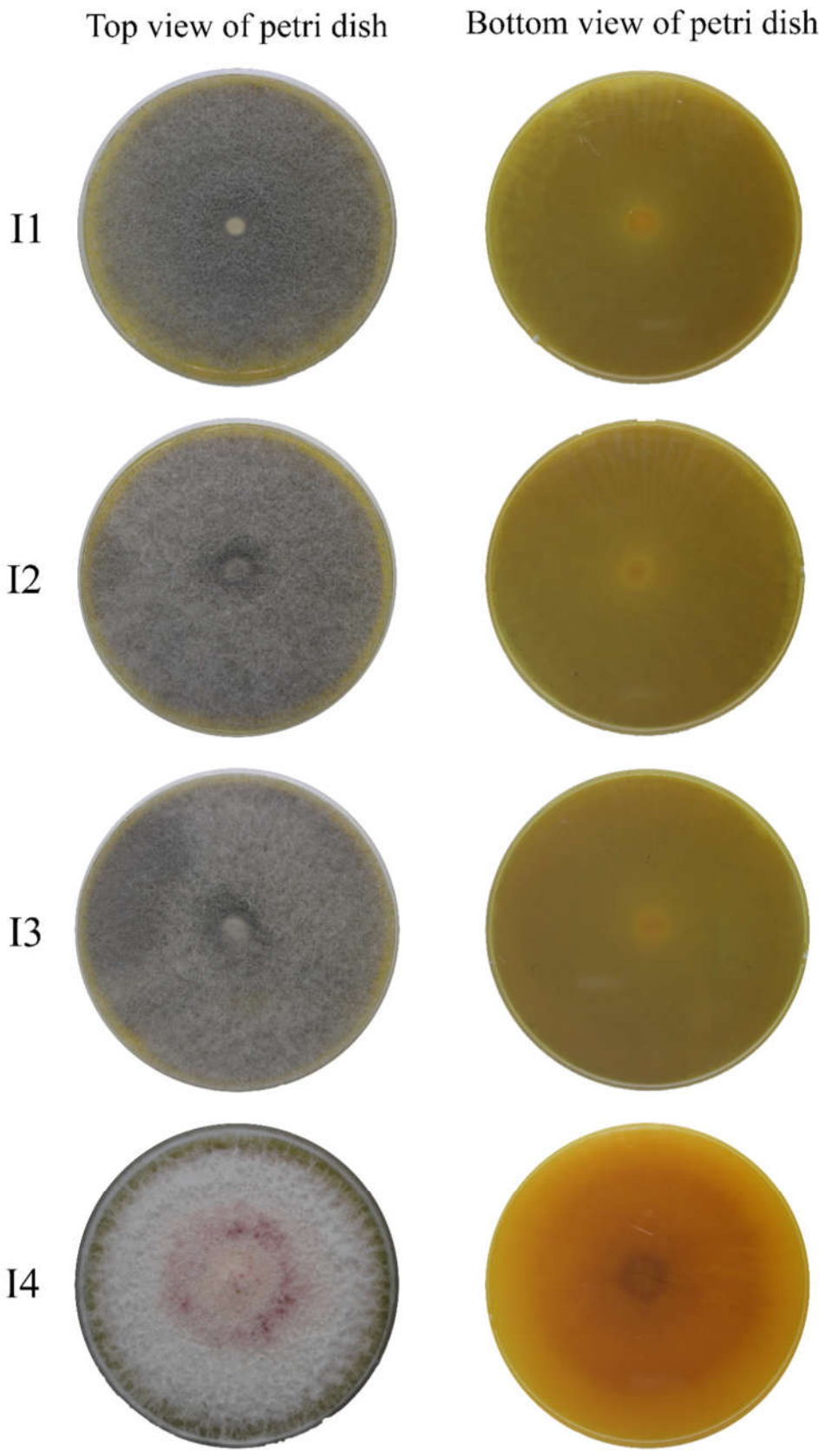

Figure 4: Photograph of the back and front of I1, I2, I3, and I4 grown on MEA (10 days, 25 °C in the dark).

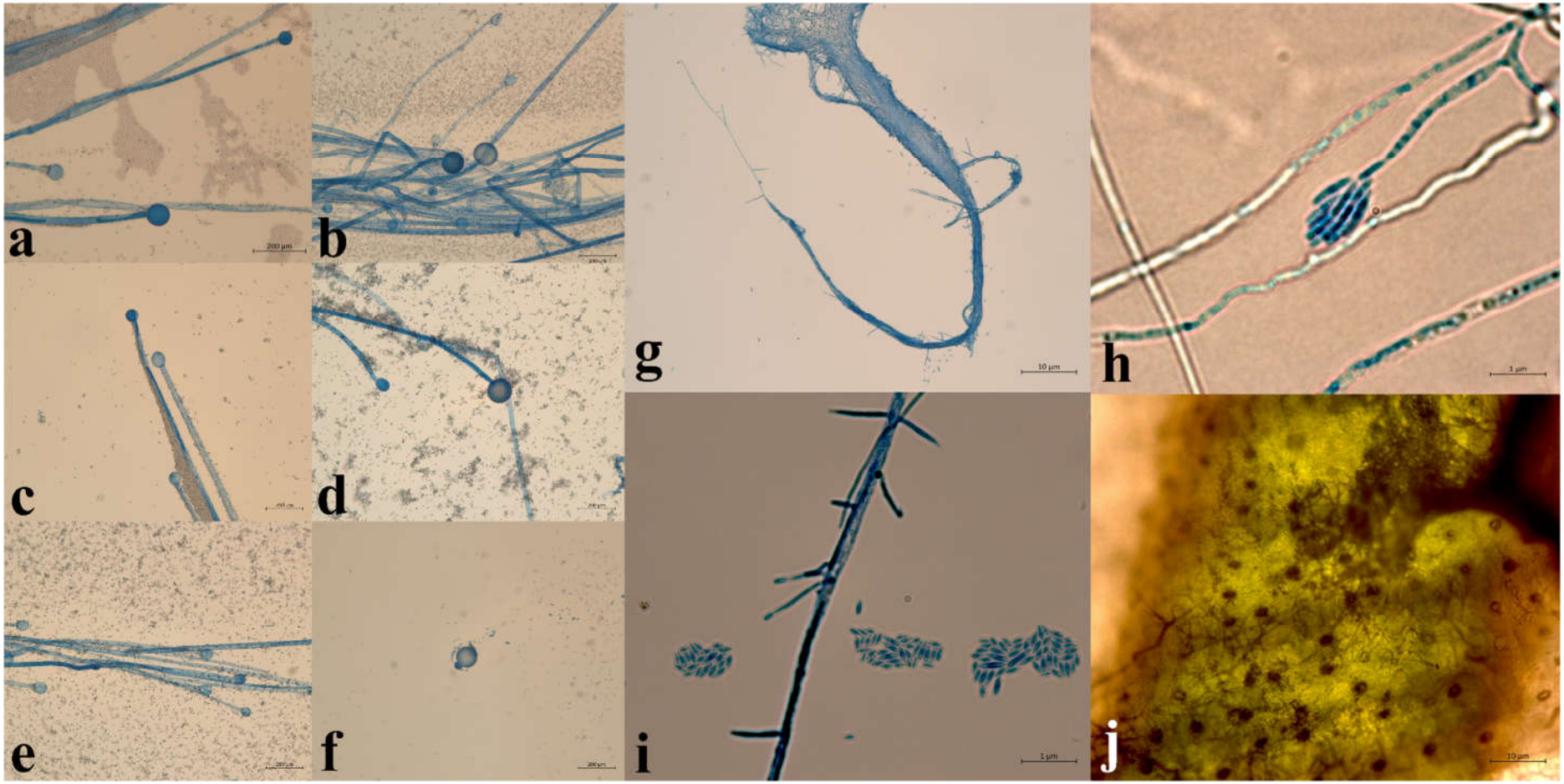

Figure 5: Microscopic morphology of the four fungal isolates harvested from the cigarette bin.

a. I1 – sporangiophore and columellae. b. I1 – sporangium and columellae. c. I2 – sporangiophore and columellae. d. I2 – sporangium and columellae. e. I3 – sporangiophore and columellae. f. I3 – sporangium and sporangia. g. I4 – hyphae morphology. h. I4 – false head containing sickle-shaped conidia with no septa. i. I4 – macroconidia and microconidia. j. I4 - false heads seen in carnation leaf. a – f: Grown for three days on MEA at 25 °C. g – j: grown for seven days on CLA at 25 °C.

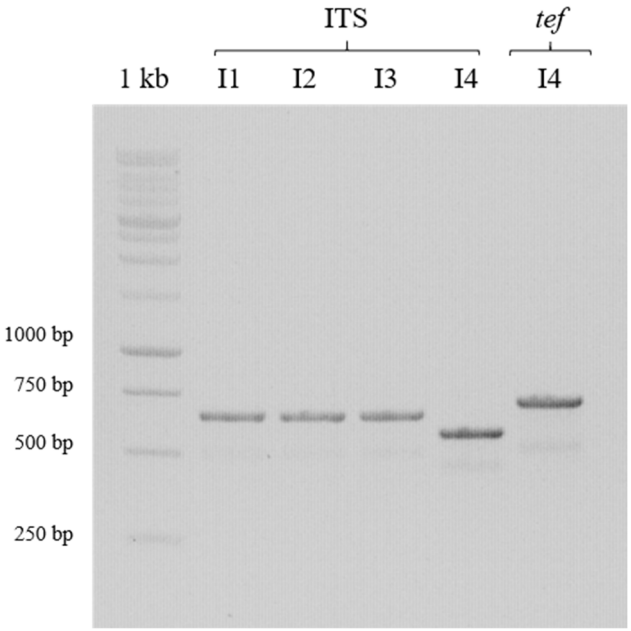

Figure 6: PCR amplification of the ITS region for all four fungal isolates, and partial amplification of the *tef1* gene for I4. PCR amplification was achieved using fungal gDNA from I1, I2, I3, and I4. The ITS amplicon for I1, I2, and I3 was ~650 bp, while the ITS and *tef1* amplicon for I4 was ~500 bp and ~700 bp, respectively.

1.3.3 Phylogenetic inference of fungal isolates harvested from the cigarette bin

Approximately 500 bp were included in the phylogenetic analysis for both the ITS and *tef1* datasets (**Table 3**). The ITS *Mucor* dataset was modelled using Tamura 3-parameter (T92) with five categories of gamma distribution. The *tef1 Fusarium* dataset was modelled using general time reversible (GTR) with five categories of gamma distribution. A bootstrap analysis of 10 000 replicates and a 70 % cutoff value for each node was applied. The highest log likelihood tree is represented with -InL = -4974.44 and -InL = -4076.11 for the *Mucor* (**Fig. 7**) and *Fusarium* (**Fig. 8**) datasets, respectively. Phylogenetic inference for I1, I2, and I3 using the ITS DNA barcode clustered all three isolates in the MCC (BS = 0.91; **Fig. 7**). There are two phylogenetic species (PS 14 and PS 15) within *M. circinelloides* f. *circinelloides*. I1 clustered in the *M. circinelloides* f. *circinelloides* PS clade 15 (BS = 0.72), while both I2 and I3 clustered in the PS clade 14 (BS = 0.97; Wagner *et al*., 2019; Walter *et al*., 2013). Alternatively, phylogenetic inference for I4 using *tef1* as the DNA barcode clustered I4 in the *Fusarium fujikuroi* species complex (FFSC; BS = 0.85). Within the FFSC I4 identified as *Fusarium proliferatum* (BS = 1.00; **Fig. 8**).

Table 3: List of species and strains included in the phylogenetic analyses.

Organism	Source	ITS[1]	Reference
Mucor abundans	[2]CBS 388.35	MH855716.1	Groenewald *et al.,* 2019
Mucor aligarensis	CBS 244.58	MH857771.1	Groenewald *et al.,* 2019
Mucor amethystinus	CBS 526.68	JN206015.1	Walther *et al*., 2013
Mucor amphibiorum	CBS 763.74	MH860895.1	Groenewald *et al.,* 2019
Mucor circinelloides f. *circinelloides*	CBS 192.68	JN205959.1	Walther *et al*., 2013
Mucor circinelloides f. *circinelloides*	CBS 121702	JN205966.1	Walther *et al*., 2013
Mucor circinelloides f. *griseocyanus*	CBS 116.08	JN206003.1	Walther *et al*., 2013
Mucor circinelloides f. *janssenii*	CBS 185.68	JN206006.1	Walther *et al*., 2013
Mucor circinelloides f. *lusitanicus*	CBS 108.17	JN205980.1	Walther *et al*., 2013
Mucor ctenidus	CBS 293.66	JN205976.1	Walther *et al*., 2013
Mucor ellipsoideus	CBS 126271	MH863952.1	Groenewald *et al.,* 2019

Mucor exponens	CBS 141.20	MH854686.1	Groenewald *et al.,* 2019
Mucor flavus	CBS 230.35	EU484282.1	Hoffmann *et al.,* 2008
Mucor fuscus	CBS 132.22	MH854718.1	Groenewald *et al.,* 2019
Mucor fusiformis	CBS 336.68	MH859152.1	Groenewald *et al.,* 2019
Mucor genevensis	CBS 114.08	EU484275.1	Hoffmann *et al.,* 2008
Mucor hiemalis	CBS 115.18	MH859259.1	Groenewald *et al*., 2019
Mucor indicus	CBS 120.08	MH854581.1	Groenewald *et al.,* 2019
Mucor laxorrhizus	CBS 143.85	MH861865.1	Groenewald *et al.,* 2019
Mucor luteus	CBS 243.35	AY243951.1	Han *et al., unpublishd*
Mucor microsporus	CBS 245.35	MH855663.1	Groenewald *et al.,* 2019
Mucor moelleri	CBS 216.27	MH854934.1	Groenewald *et al.,* 2019
Mucor mousanensis	CBS 999.70	MH860022.1	Groenewald *et al*., 2019

Mucor mucedo	CBS 109.16	MH854643.1	Groenewald *et al.,* 2019
Mucor odoratus	CBS 120.71	MH860028.1	Groenewald *et al.,* 2019
Mucor piriformis	CBS 169.25	EU484276.1	Hoffman *et al., 2008*
Mucor plumbeus	CBS 226.32	JN205916.1	Walther *et al*., 2013
Mucor prayagensis	CBS 816.70	MH859957.1	Groenewald *et al.,* 2019
Mucor racemosus f. *racemosus*	CBS 260.68	JN205898.1	Walther *et al*., 2013
Mucor saturninus	CBS 521.64	MH858502.1	Groenewald *et al*., 2019
Mucor silvaticus	CBS 249.35	MH855666.1	Groenewald *et al.,* 2019
Mucor ucrainicus	CBS 221.71	MH860077.1	Groenewald *et al.,* 2019
Mucor variisporus	CBS 837.70	MH859970.1	Groenewald *et al.,* 2019
Mucor zonatus	CBS 148.69	MH859280.1	Groenewald *et al.,* 2019
Parasitella parasitica	CBS 208.28	MH854982.1	Groenewald *et al*., 2019

Organism	Source	*tef1*[1]	Reference
Fusarium aethiopicum	CBS 123667	FJ240295.1	O'Donnell *et al*., 2008
Fusarium arthrosporioides	CBS 100485	DQ531563.1	Kulik *et al*., 2007
Fusarium asiaticum	CBS 110256	AF212450.1	O'Donnell *et al*., 2000b
Fusarium babinda	[3]NRRL 25531	MH742711.1	Jacobs-Venter *et al*., 2018
Fusarium begoniae	CBS 403.97	AF160293.1	O'Donnell *et al*., 2000b
Fusarium boothii	CBS 110250	AF212443.1	O'Donnell *et al*., 2000b
Fusarium brevicatenulatum	CBS 404.97	AF160265.1	Laraba *et. al*., 2019
Fusarium bulbicola	CBS 220.76	AF160277.1	O'Donnell *et al*., 2000b
Fusarium cerealis	CBS 110268	AF212464.1	O'Donnell *et al*., 2000b
Fusarium chlamydosporum	CBS 145.25	MN120754.1	Lombard *et al.,* 2019a
Fusarium circinatum	CBS 405.97	AF160295.1	O'Donnell *et al*., 2000b

Fusarium coeruleum	CBS 836.85	DQ164859.1	Romberg and Davis, 2007
Fusarium concentricum	CBS 450.97	AF160282.1	O'Donnell *et al*., 2000b
Fusarium concolor	CBS 961.87	GQ505674.1	O'Donnell *et al*., 2009
Fusarium culmorum	CBS 110269	AF212462.1	O'Donnell *et al*., 2000b
Fusarium denticulatum	CBS 735.97	AF160269.1	O'Donnell *et al*., 2000b
Fusarium dlaminii	CBS 175.88	AF160277.1	O'Donnell *et al*., 2000b
Fusarium domesticum	CBS 244.82	EU926287.1	O'Donnell *et al*., 2009
Fusarium equiseti	CBS 307.94	GQ505599.1	O'Donnell *et al*., 2009
Fusarium foetens	CBS 110286	GU170560.1	Migheli *et al*., 2010
Fusarium graminearum	CBS 110261	AF212455.1	O'Donnell *et al*., 2000a
Fusarium lactis	CBS 411.97	AF160272.1	O'Donnell *et al*., 2000b
Fusarium napiforme	CBS 748.97	AF160266.1	O'Donnell *et al*., 2000b

Fusarium nectrioides	CBS 176.31	EU926312.1	Schroers *et al*., 2009
Fusarium nisikadoi	CBS 742.97	AF324329.1	Baayen *et al*., 2000
Fusarium nygamai	CBS 140.95	HM347121.1	O'Donnell *et al*., 2010
Fusarium oxysporum	CBS 144135	MH485045.1	Lombard *et al*., 2019a
Fusarium penzigii	CBS 317.34	EU926324.1	Schroers *et al*., 2009
Fusarium proliferatum	CBS 217.76	AF160280.1	O'Donnell *et al*., 2000b
Fusarium pseudoanthophilum	CBS 745.97	AF160264.1	O'Donnell *et al*., 2000b
Fusarium pseudocircinatum	CBS 449.97	AF160271.1	O'Donnell *et al*., 2000b
Fusarium pseudograminearum	CBS 109956	AF212468.1	O'Donnell *et al*., 2000b
Fusarium pseudonygamai	CBS 417.97	AF160263.1	O'Donnell *et al*., 2000b
Fusarium ramigenum	CBS 418.97	AF160267.1	O'Donnell *et al*., 2000b
Fusarium redolens	CBS 530.96	AF324319.1	Baayen *et al*., 2000

Fusarium sacchari	CBS 223.76	AF160278.1	O'Donnell *et al*., 2000b
Fusarium scirpi	CBS 448.84	GQ505605.1	O'Donnell *et al*., 2009
Fusarium solani	CBS 101427	GQ505674.1	O'Donnell *et al*., 2009
Fusarium sporotrichioides	CBS 447.67	HM347118.1	O'Donnell *et al*., 2010
Fusarium thapsinum	CBS 733.97	AF160270.1	O'Donnell *et al*., 2000b
Fusarium udum	CBS 178.32	AF160275.1	O'Donnell *et al*., 2000b
Fusarium verticillioides	CBS 734.97	AF160262.1	O'Donnell *et al*., 2000b
Stachybotrys chartarum	CBS 129.13	KM231994.1	Lombard *et al*., 2015

[1] ITS: internal transcribed spacer; *tef1*: translation elongation factor-1alpha.
[2] CBS: The Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.
[3] NRRL: Agricultural Research Service Culture Collection, USA.

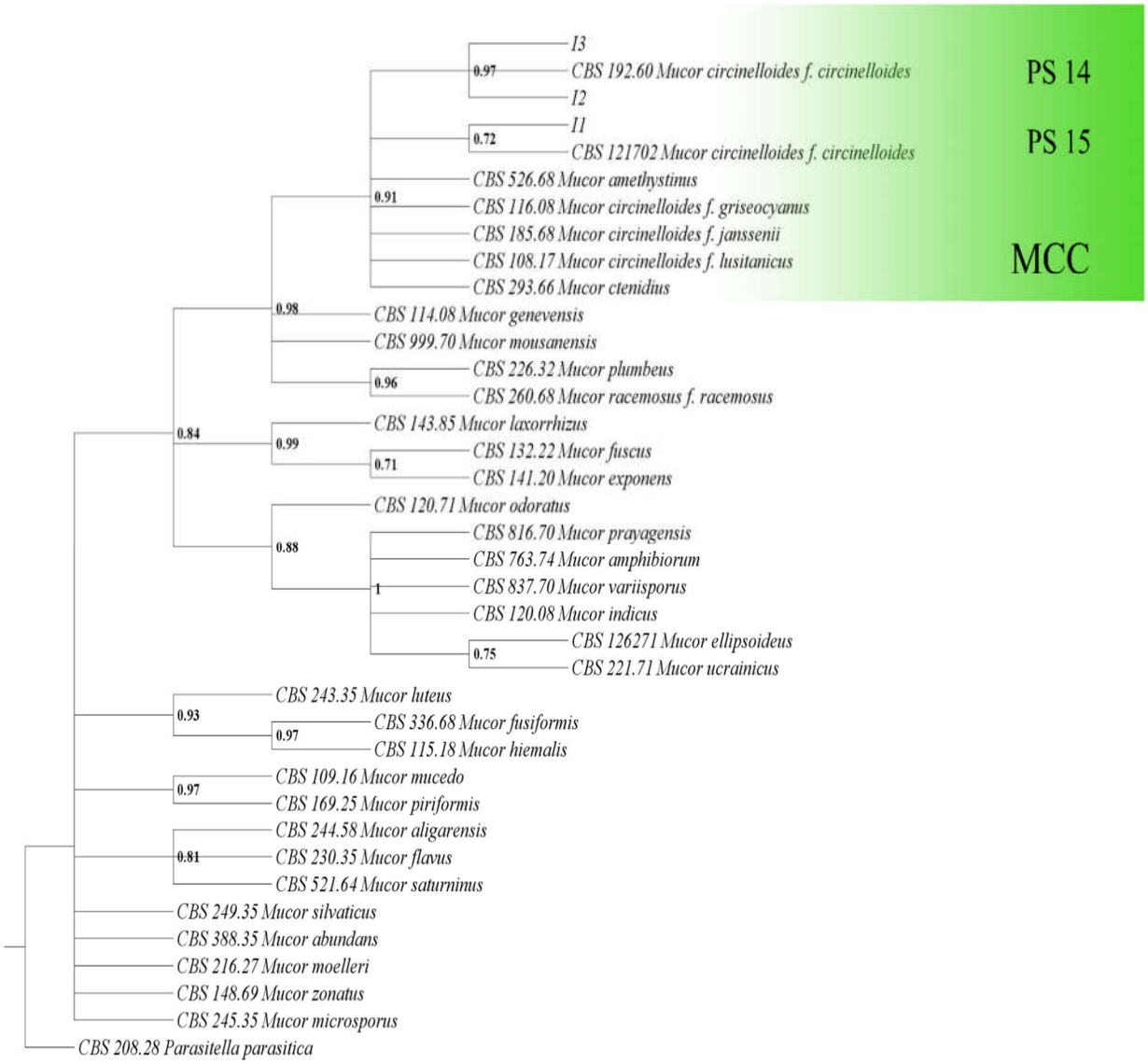

Figure 7: Maximum likelihood tree inferred from the ITS region, including the 5.8S rRNA gene sequence for I1, I2 and I3. A cutoff bootstrap value of 70 % was applied, with the decimal value indicated at the node (10 000 replicates). CBS 208.28 *Parasitella parasitica* represents the outgroup. The *Mucor circinelloides* species complex (MCC) is highlighted, indicating the two phylogenetic species PS 14 and PS 15.

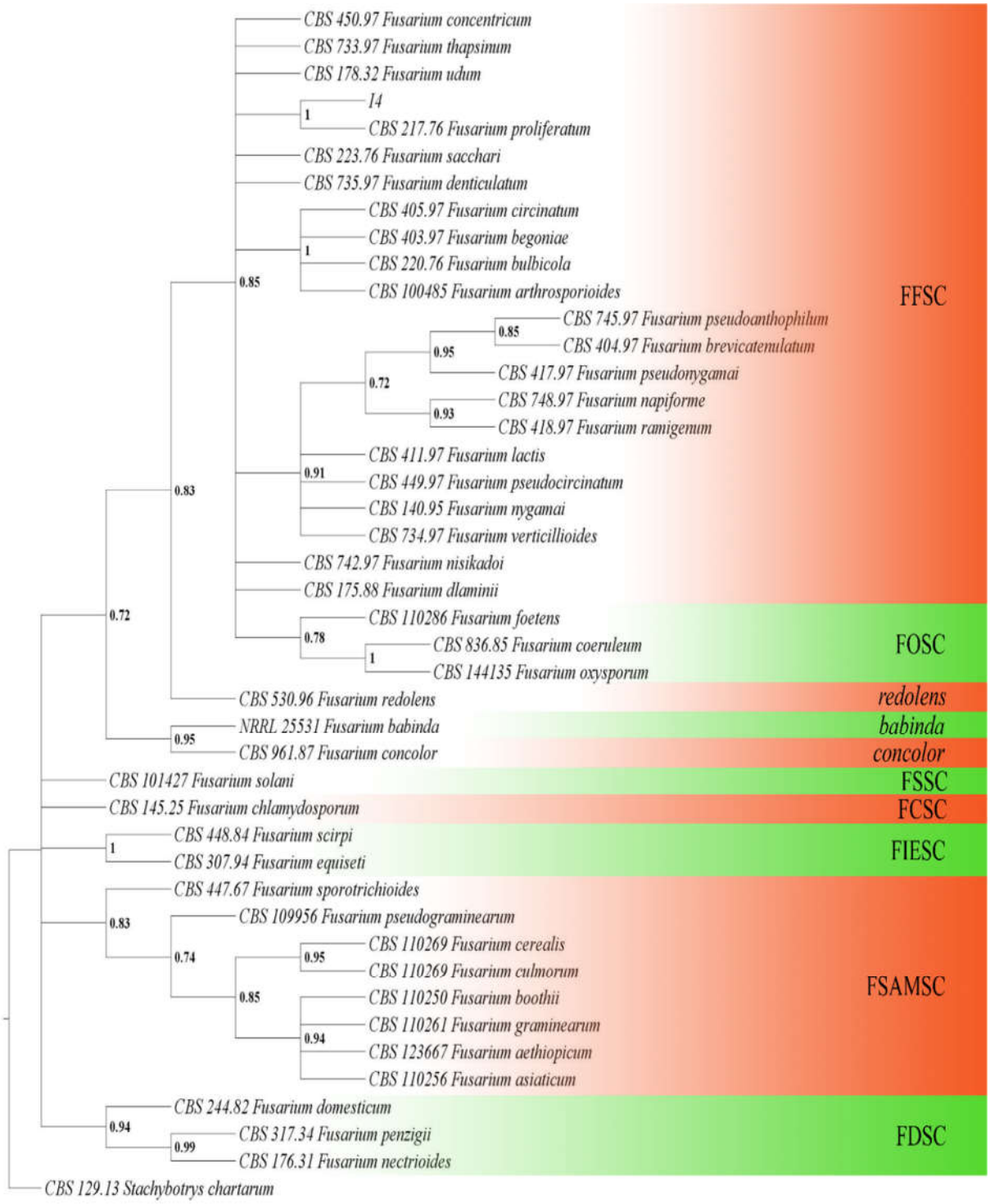

Figure 8: Maximum likelihood tree inferred from partial sequence typing of the *tef1* gene for I4. A cutoff bootstrap value of 70 % was applied, with the decimal value indicated at the node (10 000 replicates). CBS 129.13 *Stachybotrys chartarum* represents the outgroup. The following species complexes were included in the phylogenetic study: *Fusarium fujikuroi* species complex, FFSC; *Fusarium oxysporum* species complex, FOSC; *Fusarium redolens*; *Fusarium babinda*; *Fusarium concolor*; *Fusarium solani* species complex, FSSC; *Fusarium chlamydosporum* species complex, FCSC; *Fusarium incarnatum-equiseti* species complex, FIESC; *Fusarium sambucinum* species complex, FSAMSC; and *Fusarium dimerum* species complex, FDSC.

1.4 Discussion

Given the global prevalence of cigarette filter pollution and the delineation of rayon derived materials that contribute to the marine microplastic crisis, innovative research and solutions for effective waste management of cigarette filters is paramount (Cozar *et al*., 2014; Marinello *et al*., 2020; Novotny *et al*., 2009; Novotny and Slaughter, 2014; Woodall *et al*., 2014). Despite the global efforts for strict legislation regarding the use of tobacco products, the industry remains a global giant with an estimated total of 9 trillion cigarettes will be sold to the consumer market in 2025 alone (Ng *et al*., 2014; Novotny *et al*., 2009). An estimated two-thirds of smokers incorrectly dispose of their cigarette filters, resulting in trillions of smoked cigarette filters entering the environment as litter every year (WHO, 2017; Patel *et al*., 2013). Throughout the degradation process, the cigarette filter remains a vector for a myriad of toxic compounds that are harmful to a range of organisms (Moriwaki *et al*., 2009; Novotny *et al*., 2009; Novotny and Slaughter, 2014; Peppendieck *et al*., 2016; Slaughter *et al*., 2011; Wright *et al*., 2015). Additionally, as the cigarette filter degrades, thousands of microscopic cellulose acetate fibers are released into the environment contributing to the marine microplastic epidemic and deep-sea sink (Novotny and Slaughter, 2014). Cellulose acetate fibers are a ubiquitous pollutant covering 2 billion km^2 of ocean seabed and contamination within the food-chain is prevalent (Lusher *et al*., 2013; Obbard *et al*., 2014; Woodall *et al*., 2014).

Although cigarette filters are regarded as biodegradable within the scientific community, the rate of degradation can vary between different environments with estimates of 10 years before a cigarette filter is completely degraded (Bonanomi *et al*., 2015, 2020; Puls *et al*., 2010). Genomic studies focused on polluted environments have highlighted the potential genetic resourcefulness of pollution/waste (Borowik *et al*., 2017). However, genomic studies focused on the microbial ecology of smoked cigarette filters is limited (Bonanomi *et al*., 2015, 2020). The work presented here centers around a cigarette bin that was conceptualized to be inhabited by micro-organisms capable of rapidly degrading smoked cigarette filters. Subsequently, the bacterial community and potential genetic resourcefulness of the micro-organisms within the cigarette bin were investigated. With the bacterial community of the cigarette bin previously analyzed *via* 16S small subunit rRNA metagenomic sequencing, we sought to investigate the cultivatable fungal organism for a holistic analysis of the microbial ecology existing within the cigarette bin.

Species-level identification was confirmed through amplifying and sequencing specific DNA barcodes. The ITS region for all four fungal isolates was successfully amplified using the ITS4/ITS5 primer pair (**Fig. 6**; White *et al*., 1990). The ITS region as a DNA barcode for the genus *Mucor* proved highly efficient, clustering I1, I2, and I3 in the MCC (BS = 0.91; **Fig. 7**). Within the MCC, phylogenetic species (PS) are present with two major clades represented as PS 14 and PS 15 (Wagner *et al*., 2019; Walther *et al*., 2013). I1 clustered in the *M. circinelloides* f. *circinelloides* PS clade 15 (BS = 0.72), while both I2 and I3 clustered in the *M. circinelloides* f. *circinelloides* PS clade 14 (BS = 0.97; **Fig. 7**). Despite the high degree of convergence between the three *M. circinelloides* f. *circinelloides* isolates, the ITS region as a DNA barcode proved remarkably valuable, enabling species-level identification within the MCC PS clades. This is assured as the ITS region as a DNA barcode for species-level identification within *Mucorales* is well reported as an efficient DNA barcode (Kwaśna *et al*. 2006; Meyer and Grams, 2003; Schwarz *et al*. 2006; Vitale *et al*. 2012). Based on morphology alone, species-level identification within the MCC PS clades would not be possible due to cryptic speciation and similar microscopic features (**Fig. 5**).

Alternatively, the use of the ITS region for phylogenetic inference within *Fusarium* is uninformative at the species-level. This is largely due to the closely related species complexes (*F. graminerarum, F. oxysporum* and *F. fujikuroi*) sharing near identical or identical ITS rDNA alleles. The ITS region is cladistically uninformative due to the non-orthologous rDNA ITS2 types between *F. fujikuroi* and *F. oxysporum* (O'Donnell *et al.,* 1998; O'Donnell and Cigelnik *et al*., 1997; Waalwijk *et al*., 1996). Consequently, the ITS sequence information for I4 was discarded from the phylogenetic study due to the limited utility of the ITS region for species-level identification within *Fusarium*. The use of *tef1* as a standard DNA barcode for species-level identification within *Fusarium* is well accepted within the scientific community (O'Donnell *et al*., 2013; Stielow *et al*., 2015). Phylogenetic inference for partial sequence typing of *tef1* for I4 clustered within the FFSC (BS = 0. 85; formerly known as *Gibberealla fujikuroi* when teleomorphs were included in the classification of The International Code of Nomenclature), and successfully identified I4 as *F. proliferatum* (BS = 1.00; **Fig. 8**). Although species identification within the FFSC was achieved, cladistically the three American, African, and Asian clades within the FFSC were not strongly supported using *tef1* alone, which is reinforced in other studies (O'Donnell *et al*., 2010; O'Donnell *et al*., 2015). However, for the purpose of this study, *tef1* as a DNA barcode successfully separated the *Fusarium* species complexes and achieved species-level identification for I4 (**Fig. 8**). With successful DNA

barcoding and phylogenetic inference, greater access to species information for *M. circinelloides* and *F. proliferatum* is extremely relevant and insightful.

The first microscopic observations and description of *Mucor* was inscribed in 1665 by Robert Hooke (Ainsworth, 1965). To date, several hundred species have been reported forming the order *Mucorales (*from the division *Basidiomycota)*, a phylogenetically ancient group of fungi (Spatafora *et al*., 2017; Walter *et al*., 2013). *Mucor* is a polyphyletic genus with clear lineages forming species complexes. The taxonomy of the genus *Mucor* is substandard in terms of molecular phylogenetics and the phylogenetic status is controversial (Wagner *et al*., 2019; Walther *et al*., 2013; Walther *et al*., 2019; O'Donnell *et al*., 2001). Well-accepted fungal DNA barcodes such as actin, *tef1*, beta-tubulin, and calmodulin occur as multiple copies within the genus *Mucor* (Walther *et al*., 2013). The identification of *Mucor* species are mostly based on the ITS region alone, as genealogical concordance phylogenetic species recognition concept (GCPSR) is not viable (Ariyawansa *et al*., 2014; Li *et al*., 2016; Tibpromma *et al*., 2017; Wagner *et al*., 2019). The genus *Mucor* is described by 76 species, defined by several species' complexes known as *M. circinelloides, M. racemosus, M. flavus, M. hiemalis, M. fuscus, M. mucedo, M. zonatus* and *M. amphibiorum*. Within the *M. circinelloides* species complex, several distinct species or subspecies are present including *M. circinelloides* f. *lusitanicus, M. circinelloides* f. *circinelloides*, *M. circinelloides* f. *griseocyanus, M. ctenidius* and the newly amended *M. amethystinus* (Wagner *et al*., 2019; Walther *et al*., 2013; Walther *et al*., 2019).

Mostly as saprotrophs (and some instances as endophytes), *Mucor* species have been reported in soil, wet organic material, decomposing plant material, animal tissue and food spoilage (Benny *et al*., 2014; Morin-Sardin *et al*., 2017; Nout and Aidoo, 2010; Pitt and Hocking, 2009; Voigt *et al*., 2016). Some species of *Mucor* are known to infect immunocompromised patients, causing the life-threatening disease mucormycosis (Petrikkos *et al*., 2012; Roden *et al*., 2005; Wagner *et al*., 2019). In contrast, some species of *Mucor* are used in the fermentation of food products such as soy and cheeses (Hermet *et al*., 2012; Morin-Sardin *et al*., 2017). *Mucor* species have been utilized for biotechnological applications due to the wide range of growth conditions, minimal nutrient requirements, high growth rates, available genomic information, and the production of industrially relevant enzymes. At the species-level, *M. circinelloides* are a dimorphic group of fungus that have been isolated from a broad range of environments. They are considered cosmopolitan fungi; a description of prevalent fungi in terms of distribution and

ecology (de Souza *et al*., 2017; Wagner *et al*., 2019; Walther *et al*., 2019). *M. circinelloides* has been thoroughly investigated over the last several decades for industrial applications and have been reported for producing a plethora of hydrolytic enzymes, lipids, carotenoids, and ethanol, while generating a highly nutritional biomass (Rodrigues *et al*., 2019). The genome of *M. circinelloides* has been sequenced and is considered a candidate model organism for filamentous fungi. Efficient genetic markers and transformation protocols such as a leucine auxotrophic complementation system and a plasmid free CRIPSR-Cas9 system are available for *M. circinelloides* (Nagy *et al*., 2017; Ronceroet *et al*., 1989).

Alternatively, the genus *Fusarium* from the division *Ascomycota* is an infamous plant and animal pathogen causing several diseases to a myriad of hosts (Dean *et al*., 2012; Nelson *et al*., 1993; O'Donnell *et al*., 2013; Summerell, 2019). Mostly known as plant pathogens, almost 80 % of economic crops are associated with at least one *Fusarium* causing disease (Leslie and Summerell, 2006). *Fusarium* is widely distributed having pathogenic effects such as canker development in soft- and hardwood trees, vascular wilting and root rot in vegetables, and destructive head blight and root rot in several cereal grains (Goswami and Kistler, 2004; Kvas *et al*., 2008; Nyvall *et al*., 1999; Parry *et al*., 1995; Vujanovic *et al*., 2006; Wingfield *et al*., 2008). The two most important plant pathogens within *Fusarium* include *Fusarium graminearum* (causing wheat head blight) and *Fusarium oxysporum* (causing wilt and stem rot), both of which are well cited in literature and contribute to major agricultural economic losses (Dean *et al*., 2012). However, *Fusarium* species are not limited to plant-pathogenic relationships alone and expand a wide range of environments comprising of pathogens, endophytes, and saprophytes. They are cosmopolitan fungi mostly occurring in soil, plants, decaying plant material and organic matter (Abdalla *et al*., 2000; Burgess and Summerell, 1992; Jeschke *et al*., 1990; Laurence *et al*., 2015; Neish *et al*., 1983; Saha, 2002). *Fusarium* species have been reported as a human pathogen causing the life-threatening disease fusariosis (Barrios *et al*., 1990; Herbrecht *et al*., 2004; Lortholary *et al*., 2010; Nelson *et al*., 1994; Nucci and Anaissie, 2002; Summerbell *et al*., 1998; Sun *et al*., 2018; Tortorano *et al*., 2014) Furthermore, species within *Fusarium* are known to produce secondary metabolites such as trichothecenes, fumonisins, and zearalenone, all of which are mycotoxins known to have deleterious effects on animal health (Bottalico, 1998; Bottalico and Perrone, 2002; Glenn, 2007; Ross *et al*., 1990). Due to the scientific and economic importance of *Fusarium*, mycologists have highlighted the need for a well-structured phylogeny, with well-defined generic species concepts, for effective species identification and diagnosis.

For this study, we adopted the most widely accepted proposal for *Fusarium* phylogeny to date, including species concepts all defined under the genus *Fusarium*. The purpose of this study was not to evaluate the current phylogenetic state of the genus *Fusarium*, but rather conduct species identification for I4 in order to identify the cultivatable fungi harvested from the cigarette bin. First established over 200 years ago, the genus *Fusarium* has undergone dramatic changes and adaptations to taxonomic records (Summerell, 2019; Thrane and Seifert, 2000). Currently, over 300 phylogenetically distinct species exist within the genus, however, less than half are formally described. The phylogeny of *Fusarium* is contentious, with two major proposals amongst fursariologists: splitting *Fusarium* into the generic genus names *Albonectria, Cyanonectria, Geejayessia* and *Neocosmospora* and *Bifusarium* (Gräfenhan *et al*., 2011; Lombard *et al*., 2015; Nalim *et al*., 2011; Schroers *et al*., 2011), and keeping the genus name *Fusarium*, for a strongly supported polyphyletic clade comprising of species concepts (Geiser *et al*., 2013; O'Donnell *et al*., 2013). Both provide stability and a well-defined phylogeny of *Fusarium*; however, the major differences include at which point two clades become different genera. From a biotechnological perspective, *Fusarium* are well-known for the production of pigments, bioflavours, biofuel, industrial enzymes, and bioactive compounds (Abdel-Azeem *et al*., 2019; Pessôa *et al*., 2017). The genome of 68 *Fusarium* species have been sequenced and deposited in the NCBI database (Cen *et al*., 2020; Bashywal *et al*., 2017; Jeong *et al*., 2013; Niehaus *et al*., 2017; Wiemann *et al*., 2013). Additionally, transformation methods are available for *Fusarium* species such as electroporation, PEG-mediated transformation, and *Agrobacterium* transformation (Bernardi-Wenzel *et al*., 2016; de Groot *et al*., 1998; Fernandez-Martin *et al*., 2000; Garcia-Martinez *et al*., 2015; Islam *et al*., 2012).

Within the cigarette bin scenario, fungal organisms harvested from such an environment may present interesting or novel features enabling the efficient degradation of cigarette filters (Bonanomi *et al*., 2020; Puls *et al*., 2010). Fungi are known to be excellent producers of enzymes capable of degrading recalcitrant molecules/pollutants, as well as cellulose and cellulose-derived materials (Maamar *et al*., 2020; Prenafeta-Boldú *et al*., 2006; Tigini *et al*., 2009; Varadarajan and Shikha, 2014; Ventorino *et al*., 2018). Isolating and characterizing fungal species from cigarette filter waste, such as a cigarette bin or smoked cigarette filter, is however not reported in peer-reviewed scientific journals. Due to the limited research on culture-dependent approaches for fungi isolated from cigarette filter waste, culture-independent approaches were compared (Bonanomi *et al*., 2020). The study focused on relative abundance of bacteria and fungi over-time in terms of phyla found within cigarette filters

incubated in different environments (Bonanomi *et al*., 2015, 2020). The fungal community within the cigarette filters were mostly occupied by *Ascomycota* and *Basidiomycota*. The reported fungal species in terms of phyla found within decomposing cigarettes included *Chaetornium, Davidiella, Phlalophora, Aspergillus, Eurotium, Articulospora, Sclerotinia, Athrobotrys, Dactylella, Monacrosporium, Stachybotrys, Nectriaceae, Chaetomiacea, Phoma, Penicillium, Paraconiothyri, Xylaria, Amphinema, Pisolithus, Sebacinaceae, Thelephoraceae, Sporobolomyceae, Cryptococcus, Catenaria, Basidiomycota, Inocybe, Tomentella, Coprinellus, Rhizopogon* and *Lepiota*. This was achieved through extracting environmental DNA from the cigarette filters for the preparation of a library using the ITS primer pair: BITS2F/B58S3 (Bokulich and Mills, 2013; Bonanomi *et al*., 2020). Both *M. circinelloides* and *F. proliferatum* form part of the phyla *Basidiomycota* and *Nectriaceae*, respectively. This suggests that the species isolated in our study may too have inhabited the cigarette filters in the reported study (**Fig. 4**; Bonanomi *et al*., 2020). Cigarette filters incubated under laboratory conditions were mostly occupied by *Aspergillus, Eurotium,* and *Sclerotinia* with small changes over time towards *Phoma, Penicillium* and *Paraconiothyrium* (Bonanomi *et al*., 2020). Cigarette filters incubated under soil environments, the shift over time for *Basidiomycota* exceeded 80 % of all fungi. A key finding indicated that under sand dune environments, the cigarette filter was mostly occupied by several *Basidiomycota* species capable of cellulose acetate degradation regardless of the high levels of acetylation (Bonanomi *et al*., 2015, 2020). This coincides with our results as *M. circinelloides* forms part of *Basidiomycota* and therefore suggests that some species from the division could utilize cigarette filters as a means of carbon (**Fig. 7**).

The cigarette bin from which the isolates were harvested from in this study was roughly fifteen years old. The prevailing view is that the microbiota shift within the cigarette bin enabled the proliferation of a microbial community primed for cellulose acetate degradation regardless of the high degree of acetylation. The fungal isolates harvested from the cigarette bin were conceptualized to herald novel capabilities for cigarette filter degradation. Prospectively, the fungal isolates could have undergone mutational events within promiscuous ancestral enzymes for novel capabilities and phenotypic improvements towards highly acetylated cellulose. Alternatively, the fungi cultivated may have gradual genetic improvements for enhanced enzyme kinetics towards cellulose acetate as a substrate. Or perhaps, due to the genetic adaptability of fungi, through regulatory expression mechanisms, the fungi harvested may have an enhanced cellulolytic expression primed for cellulose acetate degradation. Although *M.*

circinelloides and *F. proliferatum* were not specifically reported in the aforementioned study, members of *Basdiomycota* and *Nectriaceae* were reported (Bonanomi *et al*., 2020). The family *Mucoraceae* forms the order *Mucorales* which are included in the division *Basdiomycota*. The family *Nectriaceae* from the order *Hypocreales* which is included in division *Ascomycota*. Both *M. circinelloides* and *F. proliferatum* form part of *Basdiomycota* and *Ascomycota* (*Nectriaceae*), respectively (**Fig. 7**; **Fig. 8**).

M. circinelloides and *F. proliferatum* are known to produce a complete cellulolytic cocktail enabling the utility of cellulose and cellobiose as the sole carbon substrate (Alves *et al*., 2002; Behera and Ray, 2016; Gupta and Verma, 2015; Huang *et al*., 2014; Indira *et al*., 2016; Olajuyigbe *et al*., 2016; Panagiotou *et al*., 2011; Saha, 2004; Shimonaka *et al*., 2006; Takano and Hoshino, 2012; Xiros *et al*., 2011). To date, most enzyme applications for both *Mucor* and *Fusarium* encompass the degradation of non-edible lignocellulosic biomass to produce second generation biofuel. This was explicitly shown in a number of studies using various cellulose-based substrates such as corn cob, corn starch, straw, wheat bran and brewers' grain (Ali *et al*., 2016; Almeida *et al*., 2014; Anasontzis *et al*., 2011; Baeyens *et al*., 2015; Rodrigues *et al*., 2019; Vohra *et al*., 2014; Xiros *et al*., 2011; Xu *et al*., 2015). Interestingly, an analysis of 26 strains from six *Mucor* species (including *M. circinelloides, M. hiemalis, M. genevensis, M. piriformis, M. racemosus* and *M. variosporus*), explicitly indicate that *M. circinelloides* achieved the highest production cellulases (Alves *et al*., 2002). Alternatively, many *Fusarium* species occur in close associations with plants as saprophytes/endophytes, and often exist throughout the entire life cycle of the plant (Saikkonen *et al*., 1998; Zeller *et al*., 2003). Such saprotrophic/pathogenic *Fusarium* species are known to produce cell wall degrading enzymes for nutrient assimilation (Roncero *et al*., 2003). These cell wall degrading enzymes encompass a wide range of enzymes including cellulases (Beliën *et al*., 2006; Christakopoulos *et al*., 1996; Knogge, 1996; Ruiz *et al*., 1997; Saha, 2001; Walton, 1994). Interestingly, tobacco plants and cured tobacco products are known to be inhabited by a variety of fungal species from the genus *Aspergillus, Penicillium, Phoma, Alternaria, Chaetomium, Cladosporium, Rhizopus, Fusarium, Trichoderma, Monographella, Rhodotorula, and Sporidiobolales* (Chen *et al*., 2018a, 2018b; Villemur *et al*., 2009; Welty, 1972; Yang *et al*., 2008; Zhang *et al*., 2018). More specifically, *F. proliferatum* was reported for the first time to cause leaf spot disease of tobacco plants indicating pathogenic mechanisms for degrading tobacco leaves for nutrient assimilation (Li *et al*., 2017). In this regard, remnant tobacco and toxic compounds associated with cigarette

filters may be processed by *F. proliferatum* into non harmful compounds and nutrients (Golias *et al*., 2000; Liu *et al*., 2015; Maldonado-Robledo *et al*., 2003; Wei *et al*., 2014).

Due to the known potential of *M. circinelloides* and *F. proliferatum* within the context of lignocellulose and cellulose degradation, the enzymes responsible in the catabolic pathway may be crucial within the context of cellulose acetate degradation. In this regard, *in vitro* enzyme analysis of *M. circinelloides* and *F. proliferatum* towards cellulose-based substrates may indicate potential applications for the degradation of cigarette filters. A fungal-based biorefinery for the remediation of cigarette filters may be a promising solution for cigarette filter pollution. This approach may permit the extraction of high-value products, such as bioethanol, providing valorization of cigarette filter pollution and a sustainable business model.

Chapter 2

Fungi and the filter: can a single organism host the molecular tools for the remediation of cigarette filters?

Fungi are unique in their ability to convert complex organic materials into an array of high-value products. Fungi such as yeast have impacted humans for millennia in the fermentation of simple sugars to produce beer, wine, and bread. However, filamentous type fungi have different beneficial characteristics compared to that of yeasts, and therefore have other biotechnological applications (Hyde *et al*., 2019). In fact, Scientific American published "The mycelium revolution is upon us" in 2019, which pedestals filamentous type fungi as the organism of the future. Filamentous fungi can be grown on complex organic substrates with relative ease, providing a scalable economic business model and a carbon neutral/negative outlook. Filamentous fungi can be used for the production of composite materials, high-value products, and animal feedstock (Hyde *et al*., 2019; Meyer *et al*., 2020). Furthermore, fungi are capable of adapting to certain substrate and nutrient requirements which permits a broad range of growth conditions and industrial applications. This ability to adapt and evolve to certain substrates enables fungi a greater decomposition efficiency towards available substrates (Cherry and Fidantsef, 2003; Spatafora *et al*., 2017). As fungal mycelium permeates a specific substrate, a suite of enzymes are secreted such as amylases, cellulases, inulases, lipases, pectinases and proteases. The suite of enzymes secreted degrade the complex substrate into smaller sugars, amino acids, and fatty acids for assimilation and cellular metabolism (Walton, 1994). The use of fungi for the remediation of pollutants would work in the same manner, whereby the specific enzymes secreted, degrade the complex pollutant into nutrients for cellular metabolism and growth. Effective waste management and bioremediation using fungi may be part of the solution for cigarette filter pollution. Cigarette filters are a recalcitrant material that require a diverse range of enzymes for the complete degradation into monomeric glucose (Haske-Cornelius *et al*., 2017; Puls *et al*., 2010). Fungal isolates identified and characterized for a complete degradative pathway of cellulose acetate may hold future potential for the bioremediation of cigarette filter pollution.

Cellulose acetate degradation: a holistic view on the enzymes and catabolic pathway

Cigarette filters are made from 15 000 or more cellulose acetate fibres linked together with glycerol triacetate forming a polymeric matrix (Hamzah and Umar, 2017; Robertson *et al*., 2012). The general synthesis of cellulose acetate is a two-step process involving the treatment of cellulose for complete acetylation followed by controlled deacetylation *via* hydrolysis for different commercial applications such as hygiene products, clothing manufacturing and cigarette filters (McGath *et al*., 2015). Cellulose acetate can be characterized by two major factors: the degree of acetyl substitution (DS) and the degree of polymerization (DP). The DS, the number of occurring acetyl groups per monomeric glucose, can range from 0 to 3 for varying properties such as solvent solubilities and molecular weights (Haske-Cornelius *et al*., 2017; Puls *et al*., 2010). The higher the acetyl DS of cellulose acetate, the greater the resistance to biocatalytic degradation (Ho *et al*., 1983; Samios *et al*., 1997). Cigarette filters are made from cellulose acetate with a DS = ~2.5 (25 of the 30 potential positions of ten linked glucose monomers are acetylated). The DP, the number of repeating glucose units, can range from 16 000 to 200 000, for different commercial applications and material composition. The combination of the DS and DP of cellulose acetate results in a highly crystalline, insoluble, and recalcitrant material to microbial degradation (Abrusci *et al*., 2009; Puls *et al*., 2010; Sakai *et al*., 1996).

To understand the degradation of cellulose acetate and how the DS and DP effects microbial degradation, examination of cellulose and the associated degradative pathway is crucial. Briefly, cellulose, the most abundant natural biopolymer on earth, is a complex carbohydrate consisting of 3 000 units or more of glucose linked β-1,4-glycosidic bonds forming a crystalline polymer with amorphous regions (Hon, 1994; Lynd *et al*., 2005). It is an important structural component to many plants and serves as a carbon resource to numerous micro-organisms; which use a specific suite of enzymes to degrade this complex molecule into glucose for cellular metabolism (Knogge, 1996; Roncero *et al*., 2003). This heterogeneous group of enzymes belonging to various glycoside hydrolase families consist of three major classes forming a multienzyme system called the cellulase complex: exo-(1,4)-β-D-glucanase (EC 3.2.1.91), endo-(1,4)- β-D-glucanase (EC 3.2.1.4), and β-glucosidases (EC 3.2.1.21; Baldrian and Valaskova, 2008; Kuhad *et al*., 1997; Percival Zhang *et al*., 2006). Cellulases are differentiated to other glycoside hydrolases due to the ability to hydrolyse β-1,4-glycosidic

bonds between glucosyl residues by means of a two-step acid hydrolysis mechanism (Knott *et al*., 2014). Exoglucanases initiate the cleavage of β-glycosidic bonds acting from either the reducing or non-reducing end of the cellulose chain releasing cellobiose. Endoglucanases initiate the cleavage of *O*-glycosidic bonds within the cellulose polymer, forming smaller carbohydrate fragments with reducing and non-reducing ends. While β-glucosidases are responsible for the cleavage of cellobiose into the monomeric sugar glucose (Béguin and Aubert, 1994; Yang *et al*., 2011). Synergistically, a complex interplay between the cellulase complex results in the complete degradation of cellulose into monomeric glucose. The cellulase complex is an inducible system when fungi are grown on cellulosic material, enabling the degradation of complex carbohydrates into simple sugars for cellular metabolism (Cherry and Fidantsef, 2003; Spatafora *et al*., 2017; Walton, 1994;). Consequently, the cellulase complex has been thoroughly investigated for efficient lignocellulosic biodegradation; a carbon source for secondary biofuel production (Yang *et al*., 2011). Considering the mass pollution of cigarette filters, a systematic fungal-based biorefinery could be an efficient system for the bioremediation of cigarette filters while extracting high-value compounds or producing composite materials.

Cellulose acetate is a chemical derivative of cellulose, made of acetylated glucose linked β-1,4-glycosidic bonds. In this regard, the cellulase complex is required for the cleavage of glycosidic bonds within cellulose acetate. However, deacetylation by acetyl esterase (EC 3.1.1.6) is crucial for the initiation of cellulose acetate degradation (Haske-Cornelius *et al*., 2017; Puls *et al*., 2010). The enzyme pathway required for the degradation of cellulose acetate is highlighted below (**Fig. 9**). Without considering the additional enzymatic mechanism in the degradative pathway of cellulose acetate, the material would deem non-biodegradable. Early reports on cellulose acetate biodegradation labelled the material as non-biodegradable, due to absence of acetyl esterase within the degradative biochemical pathway (Potts *et al*., 1972). Acetyl esterases have been identified to be responsible for the removal of acetyl moieties on cellulose acetate, allowing for the cellulase complex to hydrolyse the β-1,4-glycosidic bonds within cellulose. In this regard, highly acetylated cellulosic material is regarded as biodegradable within the scientific community (Bonanomi *et al*., 2020; Ho *et al*., 1983; Puls *et al*., 2010; Potts *et al*., 1972; Samios *et al*., 1997).

The rate of degradation of cigarette filters by cellulases is dramatically affected due to the steric hindrance caused by acetyl groups. Although fungal cellulases differ to bacterial cellulosomes,

from a structural perspective, both are affected by acetyl groups found within cellulose acetate (Bayer *et al*., 1998). Fungal cellulases predominately form two separate domains known as the catalytic domain and the cellulose binding domain. The catalytic domain is responsible for the cleavage of glycosidic bonds, while the cellulose binding domain consists of approximately 35 amino acids forming a linker region between the enzyme and the cellulose molecule (Bayer *et al*., 1998). Bacterial cellulosomes differ in the cellulose binding domain with a dockerin and single scaffolding cellulose binding domain (Bayer *et al*., 1994, 2004). Due to these structural restrictions, the acetyl group found on cellulose acetate sterically inhibits the binding of the cellulase complex.

The active site of the catalytic domain within exoglucanases exists within a structural tunnel whereby the enzyme progressively funnels cellulose hydrolysing the β-1,4-glycosidic bonds releasing cellobiose (Divine *et al*., 1994). With an acetyl group protruding from cellulose, the enzyme is blocked before it can continue moving along the cellulose molecule (Saake *et al*., 1998). This was shown using a mixture of cellulolytic enzymes, excluding acetyl esterases, for the degradation of three different cellulosic materials: cellulose acetate (DS = 2.5), cellophane and unbleached kraft paper (Itävaara *et al*., 1999). Due to the absence of acetyl esterases, the cellulose acetate material was not degraded, while both cellophane and kraft paper were significantly degraded (78 % and 43 %, respectively). The active site of endoglucanases is arranged in an open manner, allowing cellulose derivatives to undergo catalytic cleavage within the internal *O*-glycosidic bonds of the cellulose molecule (Spezio *et al*., 1993). The DS of cellulose acetate stereochemically inhibits the catalytic ability of endoglucanases; however, due to the open conformation of the active site of the catalytic domain, endoglucanases could prove advantageous within a cellulose acetate degradative pathway. An endoglucanase from *Trichoderma reesi* was investigated for catalytic degradation of cellulose acetate with increasing DS and DP (Saake *et al*., 1998). Using size exclusion chromatography, the degradation of cellulose acetate by the endoglucanase, free of acetyl esterase activity, was analysed. As the DS increased to 2.5, the catalytic ability of the endoglucanase was inhibited, while at DS = 0.9, the molecule was rapidly degraded.

Ultimately, screening micro-organisms for cellulases and acetyl esterases for the remediation of highly acetylated cellulose warrants further investigation (Puls *et al*., 2010). The inconsistent structure and limitations of working with insoluble cellulose has resulted in the use of carboxymethyl cellulose (CMC) as the conventional approach for screening and inducing

cellulases (Lynd *et al*., 2002). In combination with dye substrates, CMC functional plate-based screening permits a simple and cost-effective method for identifying fungal isolates with cellulolytic activity (Teather and Wood, 1982; Zhang *et al*., 2006). At the molecular level, CMC is a cellulose derivative with carboxymethyl groups (-CH_2-COOH) substituting the hydroxyl group on a glucose monomer of the cellulose backbone (Leppänen *et al*., 2019; Simon *et al*., 1998). The DS of CMC can range from 0 to 3, due to the available -OH groups found within glucose (Leppänen *et al*., 2019). As mentioned, cellulose acetate is a derivative of cellulose, except with an acetyl group (-CH_3CO) substituting the hydroxyl group on a glucose monomer of the cellulose backbone. Similar enzymatic activities would be required for the degradation of CMC and cellulose acetate (Leppänen *et al*., 2019). This is due to the requirement of esterase activity for the cleavage of side groups found on CMC and cellulose acetate, as well as the requirement of cellulolytic enzymes (Puls *et al*., 2010). Using CMC as a carbon source would induce novel cellulolytic enzymes capable of overcoming the presence of side groups found along the cellulose backbone. CMC as a carbon source induces carboxymethyl cellulases, which are predominantly endoglucanases, emphasizing the utility of CMC as a means of screening fungal isolates for cellulose acetate degrading capabilities (Payne *et al*., 2015).

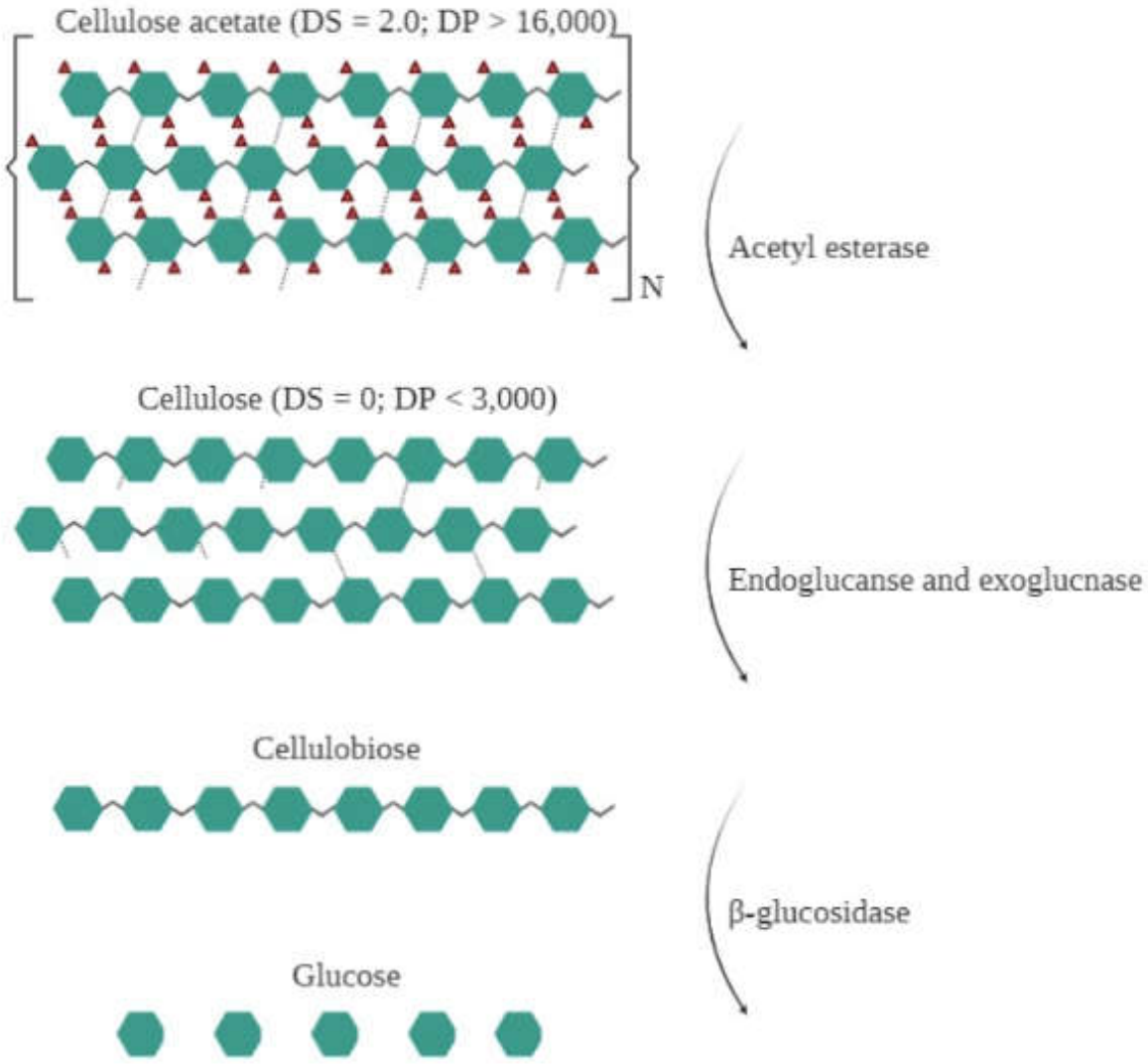

Figure 9: Cellulose acetate can be characterized by two major factors: the degree of polymerization and the degree of acetyl substitution. Here $[\ ]_N$ represents the degree of polymerization to be greater than 16,000 and DS = 2.0. At DS = 2.0, acetyl esterases exhibit activity towards the molecule allowing for the deacetylation of cellulose acetate. As the DS decreases below 2.0, both endoglucanses and exoglucanses begin to exhibit activity towards the molecule. Once the molecule is broken down into shorter carbohydrates, the molecule becomes soluble in water. As the DS = 1.0, β-glucosidases begin to exhibit activity towards the molecule, releasing glucose for cellular metabolism and growth. The synergy of these enzymes permits the successful degradation of cellulose acetate to monomeric glucose

Genetic adaptability of fungi isolated from polluted environments for an improved mycoremediation strategy

Fungi are known for their impressive ability in degrading recalcitrant materials within the environment even despite poor growth conditions and nutrient availability. The limited information on the microbial ecology and structure within polluted environments leaves gaps of information regarding the *in situ* biochemical processes that are occurring. The advent of environmental genomics and fungal genomics has aided our understanding of the microbial ecology, structure, and physiology to some degree (Cuadros-Orellana *et al*., 2013; Sharma, 2016; Srivastava, 2015). cDNA libraries have enabled a better understanding of the microbial structure and the subsequent catabolic processes responsible for the degradation of anthropogenic compounds (Testa *et al*., 2012). These culture-independent approaches involve the utility of enzymes rather than fungal whole-cell growth and biomass. The utility of enzymes negates certain limitations of fungal whole-cell growth regarding time, minimal lag phase, limited sludge/waste and better control of the overall process (Deshmukh *et al*., 2016). However, enzymes present different limitations such as shelf life, decreased stability, and exorbitant cost of enzyme production/purification. Cell-free enzyme catalysis of anthropogenic material may be ineffective depending on the structure, solubility, and physical form of the pollutant. Furthermore, the use of genetically modified micro-organisms for bioremediation within the environment raises ethical concerns and therefore should be carefully considered (Urgun-Demirtas *et al*., 2008).

From a genomic perspective, fungi isolated from a cigarette bin would herald novel and efficient cellulolytic open reading frames (ORFs) for the degradation of cellulose acetate. These adaptations permit the proliferation of fungi within a cellulose acetate-rich environment whilst surviving the toxicity of remnant tobacco. From a conceptual point of view, the adaptations required for successful cellulose acetate degradation would be expected to occur within acetyl esterases, exoglucanases, endoglucanases and β-glucosidases (Puls *et al*., 2010). These genomic clues could vary from early beneficial mutations to gradual genetic improvements. One could prospect an early beneficial mutation of an ancestral exoglucanase whereby the tunnel, where the active site is positioned, structurally widens allowing cellulose to thread through the funnel regardless of the 2.5 acetyl DS (Divine *et al*., 1994). More realistically, carbohydrate esterases and/or cellulose acetate esterases would effectively negate the initial enzymatic mechanism required for cellulose acetate degradation. Cellulose acetate

esterases have been identified and shown to deacetylate multiple carbon positions of a monomeric glucose unit within cellulose acetate (Moriyoshi *et al*., 2005; Sakai *et al*., 1996). Fungi isolated from a cigarette bin would potentially herald such genetic adaptations in order to exploit cellulose acetate as a means of carbon. Consequently, culture-dependent approaches alongside vigorous screening for esterase and cellulolytic enzyme activity would enable the identification of an efficient fungal isolate capable of degrading cellulose acetate. Furthermore, fungi can adapt to their carbon and nutrient environments, a concept of genetic adaptability, providing an enhanced strategy for the expression of such novel enzymes.

The use of fungi as a technique of bioremediation is termed mycoremediation, a cost-effective method for the degradation of anthropogenic material (Gillespie and Philip, 2013). Current applications of mycoremediation encompass a wide range of recalcitrant anthropogenic compounds such as dyes, detergents, pharmaceuticals, agricultural waste, heavy metals, herbicides, insecticides and polycyclic hydrocarbons (Azubuike *et al*., 2016; Balaji *et al*., 2014; Bhattacharya *et al*., 2011; Kapahi and Sachdeva, 2017; Khan *et al*., 2019; Kim *et al*., 2007; Lladó *et al*., 2013; Mishra and Malik, 2014; Prieto *et al*., 2011; Rodarte-Morales *et al*., 2011; Singh *et al*., 2015). This can be applied through either *in situ* remediation of polluted sites, or alternatively, controlled conditions within bioreactors (Akhtar and Mannan, 2020). Regrettable, not all fungi exude the necessary enzymes that enable the breakdown of environmental pollutants. The prevailing view of identifying and culturing fungi from polluted environments for the degradation of specific anthropogenic material remains a golden standard for bioremediation. Biostimulation and bioaugmentation are two important concepts of bioremediation that can be used to explain this phenomenon (Anderson and Juday, 2016; Silva *et al*., 2004). Biostimulation is the process of stimulating a polluted environment through changing environmental conditions in order to optimize the degradation of pollutants by native organisms. Bioaugmentation is the addition of new and/or more organisms in order to degrade pollutants within a specific environment (Fantroussi and Agathos, 2005; Hosokawa *et al*., 2009; Tyagi *et al*., 2010). In addition, autochthonous bioaugmentation takes advantage of native organisms isolated from polluted environments, which have been biochemically characterized for the degradation of specific pollutants, and are subsequently reintroduced into the environment/bioreactor after transcriptome enhancement under laboratory conditions (Ueno *et al*., 2007; Weber and Corseuil, 1994). Within the context of cigarette filters, the growth conditions and media requirements for transcriptome enhancement should promote the expression of cellulolytic enzymes. An autochthonous bioaugmentation approach for the

remediation of cigarette filters could be based on fungal inoculations of polluted environments/large-scale bioreactors, previously enriched for cellulolytic enzymes using CMC (**Fig. 10**). This approach reduces the technical expertise required for an efficient mycoremediation strategy of cigarette filters, while still benefitting from novel and beneficial genetic adaptations of fungi isolated from a cigarette bin. Yet, isolating fungi from a cigarette bin and determining the activity of specific enzymes that complement the catabolic pathway of cellulose acetate seems to be under-reported. Research investigating microbial communities found within polluted environments is contributing to advanced bioremediation technologies that aim to reduce toxic pollutants while extracting high-value products.

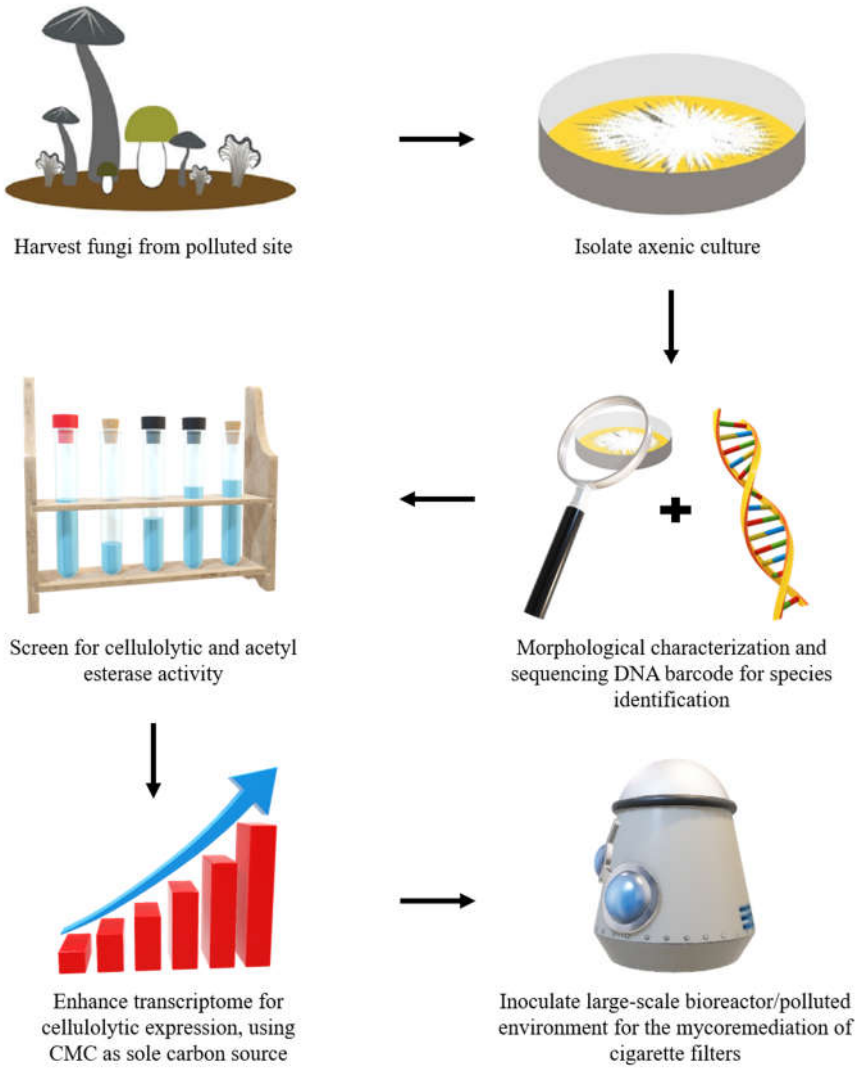

Figure 10: An autochthonous bioaugmentation approach for the remediation of cigarette filters based on a generalized strategy of isolating fungi from cellulose acetate-rich environments.

Aims and objectives

The aim of this chapter was to investigate the four fungal isolates harvested from the cigarette bin and select a candidate isolate for autochthonous bioaugmentation of cigarette filters. The four fungal isolates were screened for β-glucosidase, cellulase and esterase activity *via* functional plate-based screening in order to determine the best candidate isolate. Additionally, the four fungi were investigated for the utility/growth on cellulose and cellulose acetate cigarette filters. Based on the outcome of these two objectives, a candidate isolate was selected and investigated for a baseline activity using pNP-linked substrates *via in vitro* enzyme analysis on crude protein extracts. The following pNP-linked substrates were used in order to complement the enzymatic pathway required for cellulose acetate degradation: 4–nitrophenyl acetate, 2–chloro–4–nitrophenyl–β–cellobioside, and 4–nitrophenyl–β–D–glucopyranoside. Furthermore, the candidate isolate was enriched using CMC for enhanced cellulolytic expression primed for cellulase and acetyl esterase expression. Although comparisons in enzyme activities between the baseline (glycerol stock) and enhanced cellulolytic expression (subculture) of our candidate isolate were not conducted, the baseline activity of *F. proliferaturm* was determined using pNP-linked substrates that complements the enzymes required for cellulose acetate degradation.

2.2 Materials and methods

2.2.1 Functional plate-based screening

All four isolates were screened for β-glucosidase, cellulase and esterase activity through functional plate-based screening. Isolate spores were spotted using 10 µl of glycerol stocks on the functional plates (72 h, 25 °C in the dark for β-glucosidase and esterase activity; 120 h, 25 °C in the dark for cellulase activity). The three plates included: esculin plates (0.17 M NaCl, 1 % bacto peptone (w/v), 1.5 % agar (w/v), 0.5 % yeast extract (w/v), 0.05 % ferric citrate (w/v), 0.01 % esculin (w/v)) for screening β-glucosidase activity, CMC plates (0.5 % agar (w/v), 0.2 % gelatine (w/v), 0.188 % carboxymethylcellulose (w/v), 0.05 % K_2HPO_4 (w/v), 0.025 % $MgSO_4.7H_2O$ (w/v), 0.02 % Congo red (w/v)) for screening cellulase activity and tributyrin plates (2 % tributyrin agar (w/v), 1 % tributyrin (v/v), 0.02 % rhodamine B (w/v)) for screening esterase activity.

2.2.2 Cellulose and cellulose acetate degradation

The four isolates were investigated for cellulose and cellulose acetate degradation using cellulose and cellulose acetate filters. The observations were based on the ability of the fungal isolates to grow within the filter and use the filter as a means of carbon. Fungal spores were harvested from MEA plates (72 h, 25 °C in the dark) and normalized to 120 000 spores/ml using a hemocytometer in order to standardize the number of spores, of which 10 µl was spotted on MEA, MEA with cellulose filter and MEA with cellulose acetate filter (144 h, 25 °C in the dark). Both cellulose and cellulose acetate cigarette filters were purchased from the rolling tobacco brand Veniti®.

2.2.3 Candidate fungi I4: enhanced cellulolytic expression through CMC media enrichment

Of the four fungal isolates, only I4 indicated a clear hydrolysis zone after media enrichment, and therefore, I1, I2, and I3 were excluded from this section. Transcriptome enhancement of I4 was achieved by repeatedly subculturing on CMC media (25 °C in the dark). Initially, the time between cultivation was two to four weeks, until cultivation occurred every three days. Standard cultivating techniques were applied using dH_2O and 0.1 % Tween 80 (v/v) for spore suspension, followed by subsequent spotting of 10 µl on CMC functional plates.

2.2.4 Candidate fungi I4: culture conditions, protein extraction and *in vitro* enzyme assays for the determination of a baseline cellulolytic activity

Baseline cellulolytic enzyme activity of I4 was analyzed by growing culture inoculations in liquid LB (Luria broth; 0.17 M NaCl, 1.5 % agar, 1 % peptone, 0.5 % yeast extract; 100 rpm, 144 h, 25 °C in the dark). Protein extractions were executed by harvesting and filtering the media from the mycelia using a Whatman filter. Following this, the biomass was washed several times with sterile ddH_2O and blotted onto filter paper. The mycelia was then transferred to a 50 ml Falcon tube containing sterilized glass beads (0.5 cm in diameter) in a ratio of 1:1. After which, 4 ml of extraction buffer (50 mM HEPES-KOH pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA (ethylenediaminetetraacetic acid), 20 mM DTT (dithiothreitol), 0.1 % Triton-X 100 (v/v), 1 mM PMSF (phenylmethylsulfonyl fluoride), 50 mM sodium ascorbate, and 2 % PVP (polyvinylpyrrolidone; w/v)) was added to the Falcon tube. The 50 ml Falcon tube containing mycelia, glass beads and extraction buffer was then subjected to five repeats of vigorous shaking using a standard bench vortex (60 s), followed by 30 s on ice (5 W output). Finally, a 5 s sonication on ice before centrifugation (5 000 g, 10 min, 4 °C) in order to recover the crude protein supernatant. Protein concentrations were tested in the BioRad Bradford Protein Assay (Bio-Rad).

The pNP-linked substrates were incubated in technical repeats of three using a transparent 96-well microtiter plate (Greiner Bio-One, South Africa) with the following components: 40 µl of HEPES (100 mM, pH 7.5), 10 µl of pNP-linked substrate (0.2 mM), and 50 ul of crude enzyme lysate (30 min, 37 °C). The reactions were inhibited through the addition of 200 µl sodium carbonate (100 mM, pH 10) followed by spectrophotometric analysis at 405 nm. The following pNP-linked substrates were used: 4–nitrophenyl acetate, 2–chloro–4–nitrophenyl–β–cellobioside, and 4–nitrophenyl–β–D–glucopyranoside. Absorbances were measured at 405 nm with the VersaMax ELISA Microplate Reader and SoftMax Pro software.

2.3 Results

2.3.1 Functional plate-based screening for fungi isolated from the cigarette bin

Gross morphology for I1, I2, I3 and I4 were photographed by spotting fungal spores on LB agar plates represented as the control for the β-glucosidase functional plates (**Fig. 11**). The four fungal isolates were screened on LB agar esculin plates for the presence of β-glucosidase activity. All four fungal isolates explicitly indicated β-glucosidase activity as visualized by the brown hydrolysis zone when compared to the gross morphology of the control (**Fig. 11**). Cellulolytic fungal activity was determined based on the ability to grow on CMC plates, as well as, the formation of a clear hydrolysis zone surrounding the colony (**Fig. 11**). Initially no clear zone of hydrolysis was present for all four fungal isolates, however after repeated subculturing, *F. proliferatum* indicated a prominent hydrolysis zone (**Fig. 13**). Esterase fungal activity was determined by spotting spores onto tributyrin plates and evaluated for a clear hydrolysis zone (**Fig. 11**). All four fungal isolates were able to grow on the tributyrin plates however, the presence of a hydrolysis zone was unclear.

2.3.2 Cellulose and cellulose acetate degradation

Although the three *M. circinelloides* f. *circinelloides* grew prominently on the MEA plates, little to no growth within both the cellulose and cellulose acetate filters occurred (**Fig. 12**). The growth of *F. proliferatum* was comparably different with successful growth within the cellulose and cellulose acetate filters and consequently an indication of assimilating carbon from the cigarette filters. Evidently, *M. circinelloides* f. *circinelloides* seemed to be too large to grow within the porous matrix of the filters while *F. proliferatum*, being 10-fold smaller, was fully capable of growing between both filters (**Fig. 5**).

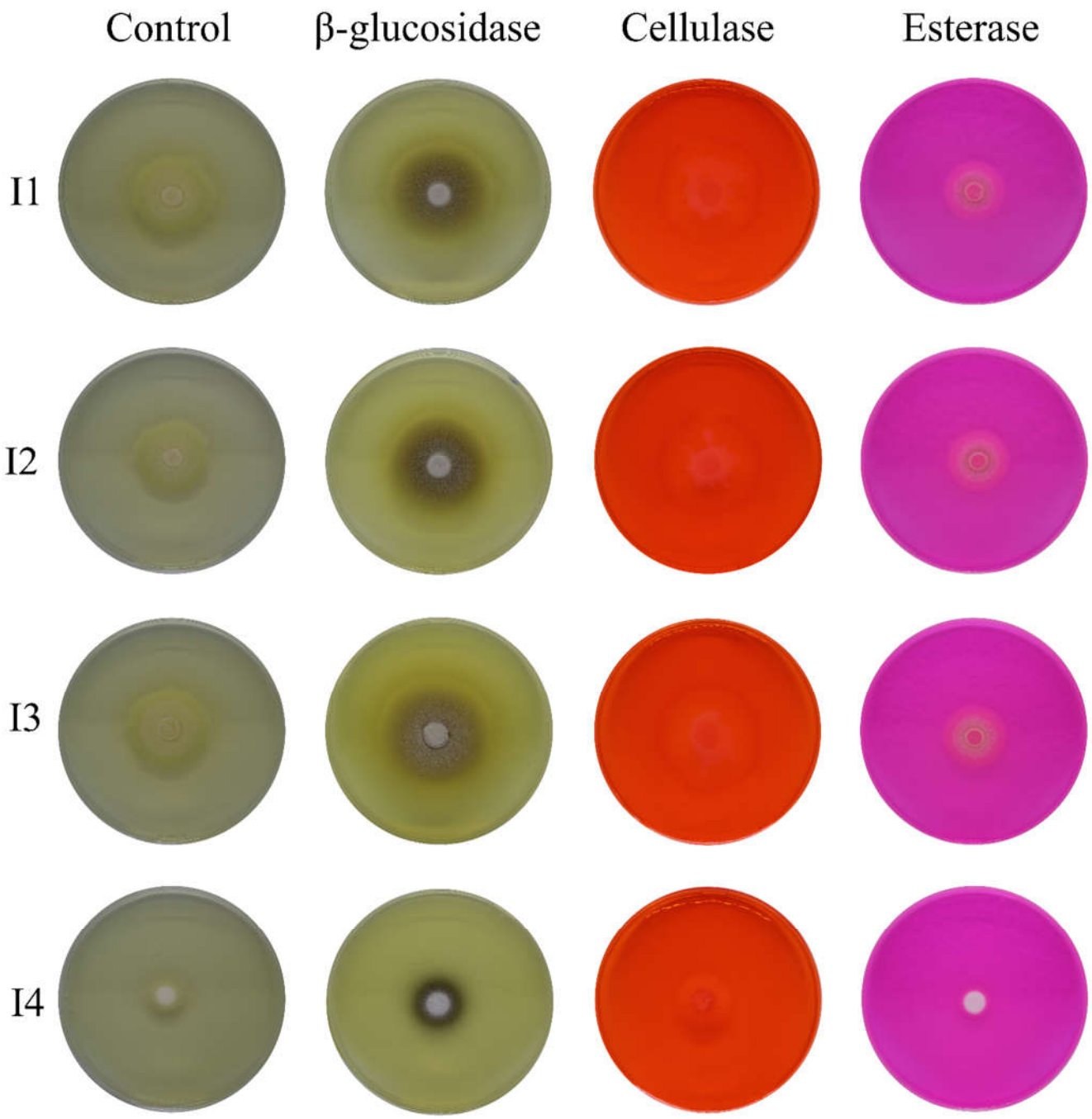

Figure 11: Functional plate-based screening for β-glucosidase, cellulase and esterase enzyme activity for I1, I2, I3, and I4. Gross morphology on LB media is represented by the control column. Esculin LB plates were used for β-glucosidase screening, with positive enzyme activity indicated by the presence of a brown halo. CMC plates were used for cellulase screening, with positive enzyme activity indicated by the presence of a clear zone. Tributyrin plates were used to screening for esterase activity, indicated by the presence of a shiny halo. All images were photographed at 72 h, except for the CMC plates which were photographed at 120 h.

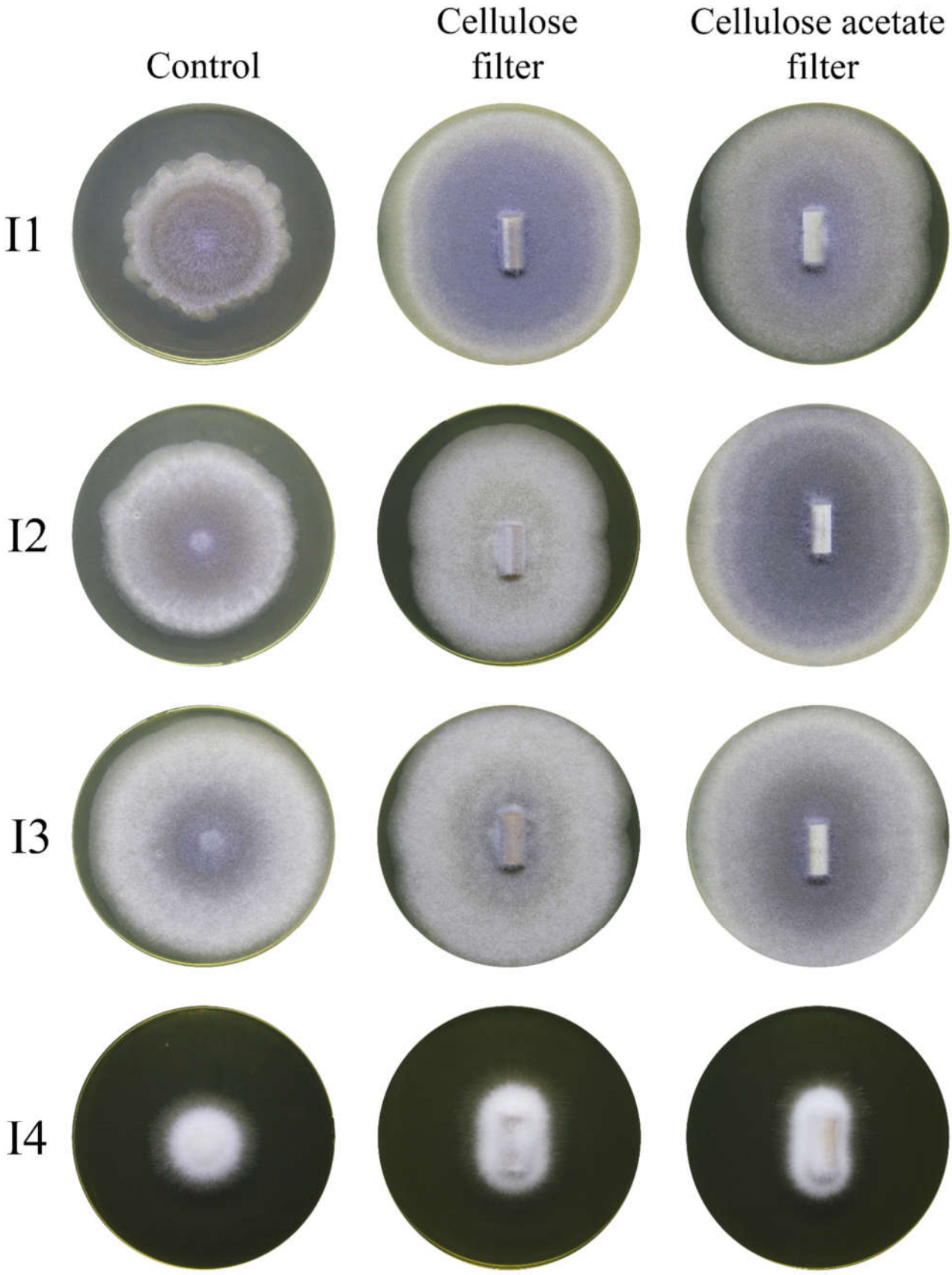

Figure 398: Investigating the growth/utility of cellulose and cellulose acetate cigarette filters. The spores were normalized to 120 000 spores/ml with 10 µl spotted onto the filter (MEA; 144 h, 25 C° in the dark).

2.3.3 Candidate fungi I4: enhanced cellulolytic expression after subculturing on CMC

After several months of subculturing I4 on CMC, *F. proliferatum* displayed a distinct genetic adaptability for the utility of CMC as a carbon source. A qualitative comparison for the genetic adaptability of I4 was based on functional plate-based screening cryogenic and enhanced cellulolytic expression spores grown on CMC (72 h, 25 °C in the dark). The criteria for successful cellulolytic enzyme activity was based on the size of the hydrolysis zone and relative growth. Based on this criteria, high levels of cellulases were produced, indicating a large hydrolysis zone, and a relatively greater biomass compared to spores from cryogenic storage (**Fig. 13**). The use of a hemocytometer was unnecessary for this experimental section as relative spore count would not induce greater cellulolytic expression or improve relative growth rates.

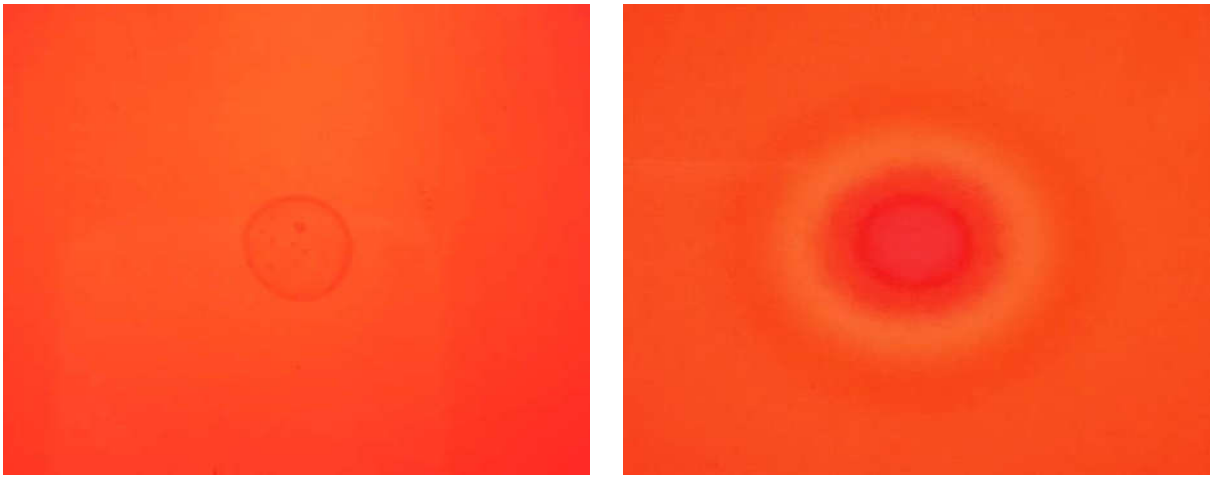

Figure 13470: I4 spotted on CMC plates supplemented with Congo red dye (72 h, 25 °C in the dark). The left image represents spores from cryogenic storage. The right image represents the enhanced cellulolytic spores from repeated subculturing.

2.3.4 Candidate fungi I4: *In vitro* enzyme assays for baseline activity using pNP-linked substrates complementing the metabolic pathway required for cellulose acetate degradation

For the determination of a holistic baseline enzyme activity for I4, culture inoculations were grown in liquid LB, a peptide- and amino-acid-rich medium. Crude protein extracts were tested on the following pNP-linked substrates: 4–nitrophenyl acetate, 4–nitrophenyl–β–D–glucopyranoside, and 2–chloro–4–nitrophenyl–β–cellobioside (**Fig. 14**). The utility of these three substrates was an attempt to complement the metabolic pathway required for cellulose acetate degradation. The mean baseline enzyme activity for the substrate 4–nitrophenyl acetate was 157.9 nkat/mg of protein, with a standard deviation of 26.0 nkat/mg of protein. The mean baseline enzyme activity for the substrate 4–nitrophenyl–β–D–glucopyranoside was 115.7 nkat/mg of protein, with a standard deviation of 1.4 nkat/mg of protein. The enzyme activity for the substrate 2–chloro–4–nitrophenyl–β–cellobioside was not determinable due to insignificant absorbance values.

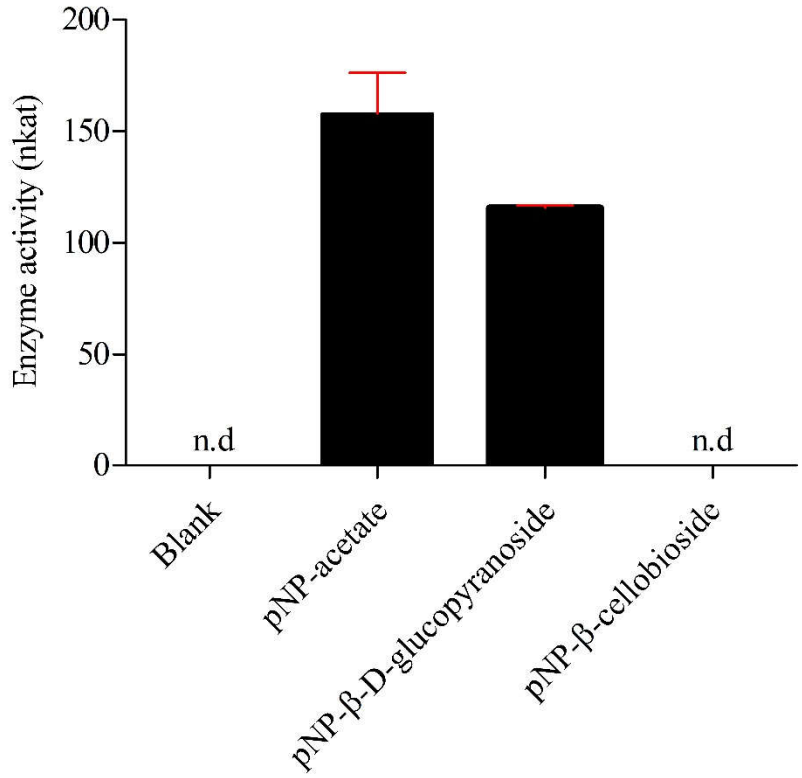

Figure 14551: Crude protein extracts from I4 grown in LB media for a baseline assessment using the synthetic chromogenic substrates 4-nitrophenyl acetate, 4-nitrophenyl-β-D-glucopyranoside, and 2-chloro-4-nitrophenyl-β-cellobioside. The crude protein extracts (50 µl) were incubated with 0.2 mM of pNP-linked substrates (10 µl) and 100 mM HEPES (40 µl) for 30 min at 37 °C. The pNP-linked substrates were incubated in technical repeats of three using a transparent 96-well microtiter plate. The reaction was inhibited through the addition of 100 mM sodium carbonate (200 µl) followed by spectrophotometric analysis at 405 nm.

2.4 Discussion

Within the last several years alone, scientific research has indicated a growing concern for cigarette filter pollution and the associated environmental effects (Araújo and Costa, 2019a, 2019b; Bonanomi *et al*., 2020; El Hadri *et al*., 2020; Green *et al*., 2019, 2020; Kataržytė *et al*., 2020; Kurmus and Mohajerani, 2020; Mansouri *et al*., 2020; Ruprecht *et al*., 2017; Torkashvand and Farzadkia, 2019; Torkashvand *et al*., 2020; Wright *et al*., 2015). Cigarette filter pollution is a global issue compounded by the prevalence of smokers and their behavior towards social acceptance of littering (Ng *et al*., 2014; Patel *et al*., 2013; Rath *et al*., 2012). The need for effective waste management and recycling solutions for cigarette filter pollution is paramount. Various solutions for recycling cigarette filters have been proposed (Marinello *et al*., 2020; Torkashvand and Farzadkia, 2019). Most solutions focus on the valorization of cigarette filters through the production of bricks, asphalt, vector controls, activated carbon, biofilms, paper, sound absorbents, metal corrosion inhibitors, and supercapacitors (Marinello *et al*., 2020; Torkashvand and Farzadkia, 2019). Biotechnological approaches within the realm of molecular biology are however lacking.

Considering the high content of cellulose within cigarette filters and the quantity of cigarette filter pollution, cigarette filters may offer an alternative source input for the production of bioethanol (Abu-Danso, *et al*., 2019; Sarkar *et al*., 2012). The use of cellulose within lignocellulosic waste is well established for the production of bioethanol. Second generation biofuels generally follow a two-step process of degrading lignocellulose into glucose followed by facile fermentation of glucose into ethanol (Anwar *et al*., 2014; Berlemont, 2017; Chen *et al*., 2018; Li *et al*., 2019). This can be incorporated into a one-step process for degradation and fermentation of lignocellulosic material using whole-cell inoculations (Molaverdi *et al*., 2019; Qiu *et al*., 2017). Lignocellulose comprises of lignin and hemicellulose, both of which are recalcitrant to enzymatic saccharification. In this regard, cellulose acetate may be a potentially improved alternative for the production of bioethanol (Abu-Danso *et al*., 2019; Cai *et al*., 2016; Molaverdi *et al*., 2019). A fungal-based biorefinery for the remediation of cigarette filters while extracting high-value products, such as bioethanol, feeds into a sustainable economic model. The generalized strategy of isolating, identifying, and characterizing fungi harvested from a cellulose acetate rich environment could provide effective solutions for cigarette filter pollution. Expanding on this generalized strategy of isolating, identifying and characterizing fungal isolates for cellulose acetate degrading capabilities, functional plate-based screening is

a cost effective and high-throughput method for identifying fungi with specific enzymatic capabilities (Coronado-Ruiz *et al*., 2018; Gohel *et al*., 2014; Johnsen and Krause, 2014; Meddeb-Mouelhi *et al*., 2014; Venkatesagowda *et al*., 2012).

In order to complete the enzyme pathway required for the degradation of cellulose acetate, multiple functional plate-based recipes were used to screen the fungal isolates. The four fungal isolates were screened for β-glucosidase, cellulase and esterase activity (**Fig. 11**); the enzymatic pathway required for cellulose acetate degradation (**Fig. 9**). All four fungal isolates indicated β-glucosidase activity through the formation of a brown halo when compared to the control (**Fig. 11**). The principle of the esculin plates for screening β-glucosidase activity is based on the hydrolysis of esculin, by β-glucosidases, releasing glucose and esculetin. The latter then binds to iron ions present within the media forming a brown halo representing β-glucosidase activity (Veena, 2011). This was to be expected, as fungi are well known for the production of β-glucosidases in order for glucose assimilation and is consolidated by the functional plate-based screening (**Fig. 11**; Divine, 1994). More so, β-glucosidases would be crucial for the complete degradation of cellulose acetate into monomeric glucose due to the nutritional limitations of a cigarette filter (Banonomi *et al*., 2020). All four fungal isolates were screened for cellulase activity using CMC functional plates (**Fig. 11**). For CMC functional plates, the formation of a clear hydrolysis zone is an indication of cellulolytic activity. Congo red exhibits a strong binding affinity towards large polysaccharides forming a red pigmentation through the media (**Fig. 11**). As cellulases degrade the polysaccharide within the media (CMC), Congo red exhibits a very weak binding affinity to the monosaccharides released, resulting in a clear hydrolysis zone (Gohel *et al*., 2014). All four isolates were fully capable of growing on CMC, suggesting the presence of inherent cellulases. Initial observations indicated that none of the isolates formed a clear hydrolysis zone. After subculturing the fungal isolates on CMC, I4 begun to elicit a clear hydrolysis zone and an increased growth rate. After several months, the enhanced cellulolytic expression of I4 drastically improved the utility of CMC as a carbon source, indicating a strong genetic adaptability towards the degradation of cellulosic material. Spores harvested from the subcultured of I4 grew much quicker and indicated a definite clear hydrolysis zone compared to spores from cryogenic storage (**Fig. 13**). Functional plate-based screening for esterase activity using tributyrin plates was unclear (**Fig. 11**). Interestingly, the dimorphic nature of the *M. circinelloides* f. *circinelloides* was induced on tributyrin with both filamentous and yeast growth present. In parallel with the functional plate-based screening, the isolates were screened for the utility of cellulose and cellulose acetate cigarette filters with

respect to the ability of the fungal isolates to grow within the filter and use the filter as a means of carbon (**Fig. 12**). I4 was receptive to both cellulose and cellulose acetate cigarette filters with relatively better growth within the cigarette filter and utility of the cigarette filter as a source of carbon.

The remediation of cigarette filter pollution requires extensive consideration in multiple facets. Firstly, due to the high acetyl DS, molecular weight, crystallinity, insolubility and physical form of cigarette filters, cell-free biocatalysis may be unfeasible (Hamzah and Umar, 2017; Haske-Cornelius *et al*., 2017; Puls *et al*., 2010; Robertson *et al*., 2012; Sakai *et al*., 1996). Considering the limited shelf life, decreased stability, and exorbitant cost of enzyme production/purification, cell-free biocatalysis limits the economic feasibility for the bioremediation of cigarette filter pollution. The use of whole-cell fungal growth provides valorisation opportunities from cigarette filter pollution in the form of high-value products such as bioethanol. Secondly, novel enzymes from fungi, heterologously expressed in bacteria, are limited to bioreactors due to strict policies and legislation regarding the use of genetically modified bacteria for *in situ* bioremediation (Urgun-Demirtas *et al*., 2008). Restricted to bioreactors, a bacterial expression system with multiple heterologously expressed ORFs for a complete catabolic pathway of cellulose acetate would innately complicate the system. Considering the plethora of toxic compounds within a cigarette filter, a bacterial expression system would need multiple mechanisms to deal with this alone. Due to the origin of the fungal isolates harvested, innate mechanism for the degradation/bioaccumulation of toxic compounds and heavy metals may be available from a genomic perspective (Cui *et al*., 2017; Zhang *et al*., 2017). Thirdly, due to the genetic adaptability of I4 towards the utility of CMC as the carbon source (**Fig. 14**), as well as the successful functional screening (**Fig. 12**; **Fig. 13**), the focus of the study centred around I4. The innate plant-pathogenic mechanisms and cell wall degrading enzymes would be available for *F. proliferatum* that could enable the isolate the ability to degrade remnant tobacco and the cellulose acetate-based cigarette filter. Ultimately, mycoremediation using whole-cell growth of cellulose acetate degrading fungi provides valorisation opportunities from cigarette filter pollution while remaining relatively simple, inexpensive, and environmentally friendly.

Protein extractions from I4, grown in liquid LB, were therefore tested *in vitro* for cellulolytic and acetyl esterase enzyme activity. This was achieved using the following pNP-linked substrates; 4–nitrophenyl acetate, 4–nitrophenyl–β–D–glucopyranoside, and 2–chloro–4–

nitrophenyl–β–cellobioside (**Fig. 14**). The principle of pNP-linked substrates for *in vitro* enzyme analysis is based on the chromogenic molecule para-nitrophenyl (pNP). pNP is a transparent molecule when bound to a substrate and a bright yellow molecule when unbound to a substrate (Bessey *et al*., 1946; King and Nicholson, 1939). When a specific enzyme acts on the pNP-linked substrate, the substrate is cleaved, releasing pNP causing a shift in absorbance. This change in absorption can be measured spectrophotometrically at an absorbance wavelength of 405 nm and compared to a set of standards. Due to the 1:1 stoichiometric ratio of pNP and substrate, one katal is the enzyme activity defined by the conversion of one mole of substrate per second at 37 °C in pH 7.5 HEPES buffer. The mean baseline activity of crude extracts from I4 was 157.9 nkat/mg of protein and 115.7 nkat/mg of protein for 4–nitrophenyl acetate and 4–nitrophenyl–β–D–glucopyranoside, respectively. There was no measurable shift in absorbance using the pNP-linked substrate 2–chloro–4–nitrophenyl–β–cellobioside from the baseline protein extracts and therefore the enzyme activity was non determinable.

Although 4–nitrophenyl acetate is a suitable substrate for the determination of acetyl esterase activity, the substrate is susceptible to other enzymatic activities such as, α-chymotrypsin, acetylcholinesterase and 3-phosphoglyceraldehyde dehydrogenase and certain classes of carbonic anhydrases (Anderson *et al*., 1994; Park *et al*., 1961; Prabhu *et al*., 2009; Taylor *et al*., 1961; Wanjari *et al*., 2012). The latter two enzymes are ubiquitous in fungi and play an important role in cellular metabolism and growth. The enzyme 3-phosphoglyceraldehyde dehydrogenase catalyzes the oxidation of 3-phosphoglyceraldehyde to 1,3-diphosphoglyceric acid, the sixth step of glycolysis, while carbonic anhydrases are responsible for the interconversion of carbon dioxide and carbonic acid (Elleuche and Pöggeler, 2009; White and Garcin, 2017). Enzyme activity towards 4–nitrophenyl acetate is not solely contributed by acetyl esterases due to the use of crude protein extracts from I4 (**Fig. 14**). Nevertheless, esterases from various fungi have been analyzed using 4–nitrophenyl acetate for the *in vitro* analysis of acetyl esterase activity from crude and purified protein extracts. A broad overview of *in vitro* enzyme activity using 4–nitrophenyl acetate for the determination of acetyl esterase activity from different fungi indicate a range of acetyl esterase activities from 2.3 nkat/mg of protein to ~45000 nkat/mg of protein (Blum *et al*., 1999; Christakopoulos *et al*., 1991; Ghatora *et al*., 2006; Luo *et al*., 2012; Luo *et al*., 2014; Pereira *et al*., 2013; Thiele and Rehm, 1979; Venkatesagowda *et al*., 2012).

Within the genus *Fusarium*, esterase activity has been reported in *Fusarium* sp., *F. sambucinum*, *F. torulosum*, *F. sarcochroum*, *F. bactridioides*, *F. oxysporum*, *F. graminearum*, *F. solani*, *F. lini* and *F. proliferatum* (Christakopoulos *et al*., 1991; Donaghy and Mckay, 1992; Hawthorne *et al*., 2001; Luo *et al*., 2014; Shin and Chen, 2006; Skovgaard and Rosendahl, 1998; Szécsi *et al*., 1995; Thiele and Rehm, 1979; Topakas *et al*., 2003; Venkatesagowda *et al*., 2012). Studies using the synthetic substrate 4–nitrophenyl acetate for determination of acetyl esterase activity have only been determined for *Fusarium* sp. with 45009 nkat/mg of protein, *F. oxysporum* with 7.1 nkat/mg and 556 nkat/mg of protein, and *F. lini* with 23554.7 nkat/mg of protein (Christakopoulos *et al*., 1999; Luo *et al*., 2014; Thiele and Rehm, 1979; Venkatesagowda *et al*., 2012). Interestingly, intracellular and extracellular protein analysis of *Fusarium* species indicate esterase activity within the intracellular protein for *F. sambucinum*, *F. torulosum*, *F. sarcochroum*, *F. bactridioides*, and *F. oxysporum* while *F. graminearum*, *F. oxysporum*, *F. solani*, *F. lini and F. proliferatum* indicate esterase activity within the extracellular protein (Christakopoulos *et al*., 1999; Donaghy and Mckay, 1992; Hawthorne *et al*., 2001; Luo *et al*., 2014; Shin and Chen, 2006; Skovgaard and Rosendahl, 1998; Szécsi *et al*., 1995; Topakas *et al*., 2003; Thiele and Rehm, 1979; Venkatesagowda *et al*., 2012). Our results from the baseline assay indicate that acetyl esterase activity was present in the intracellular protein of *F. proliferatum* with an activity of 157.9 nkat/mg of protein towards 4–nitrophenyl acetate.

Although the enzyme analysis for acetyl esterase activity within the extracellular fluid was not conducted, future studies should include both intracellular and extracellular enzyme analysis. Fungi secrete a suite of extracellular enzymes for the degradation of complex molecules within the environment for the assimilation of carbohydrates, amino acids and fatty acids. Investigating the extracellular protein extract alongside the intracellular protein extract would provide a holistic protein analysis of *F. prolierfatum*. Many *Fusarium* species such as *F. proliferatum* exhibit an inherent esterase activity, which was confirmed in the *in vitro* enzyme assay (**Fig. 14**). Considering the plant pathogenic nature of *Fusarium* species, the virulence factor has been linked to cutinase, lipase and esterase activity (Kolattukudy *et al*., 1995; Kolattukudy, 2002; Rocha *et al*., 2008). For the mycelia to penetrate the cuticle of plants, these enzymes act on polyesters consisting of hydroxy and epoxy fatty acids which form the cutin. Within the context of a cigarette bin, these inherent enzyme activities of *F. proliferatum* may be available as ancestral or promiscuous enzymes capable of mutational events for novel

enzyme capabilities for the deacetylation of short chain acetyl groups on cellulose acetate (Guzmán *et al*., 2019).

From a cellulolytic perspective, the substrates 4–nitrophenyl–β–D–glucopyranoside and 2–chloro–4–nitrophenyl–β–cellobioside were tested using the intracellular crude protein from *F. proliferatum*. The baseline enzyme activity for *F. proliferatum* towards 2–chloro–4–nitrophenyl–β–cellobioside resulted in no measurable shift in absorbance. The enzyme activity of the endo-1,4-β-glucanases from *F. proliferatum* towards the substrate 2–chloro–4–nitrophenyl–β–cellobioside was non-determinable. This was expected for the baseline activity of *F. proliferatum* as LB is a peptide- and amino-acid-rich medium that would not induce endo-1,4-β-glucanases. When grown in the presence of cellobioside, the cellulase complex is induced causing *F. proliferatum* to indicate an enzyme activity of 7101 nkat/mg of protein (Gao *et al*., 2012). A broad overview of *in vitro* enzyme activity using 4–nitrophenyl–β–D–glucopyranoside from different fungi indicate a range of β-glucosidase activity from 12.4 nkat/mg to 23671 nkat/mg of protein (Amouri *et al*., 2006; Daroit *et al*., 2008; Dikshit and Tallapragade, 2015; Joo *et al*., 2009; Kaur *et al*., 2007; Karnchanatat *et al*., 2007; Valaskova and Baldrian, 2006; Xie *et al*., 2004; Zhao *et al*., 2013). Using the substrate 4–nitrophenyl–β–D–glucopyranoside for the determination of β-glucosidase activity for *Fusarium* species have been determined for *F. oxysporum* reaching up to 60 nkat/mg of protein, *F. verticilliodes* with 601.9 nkat/mg of protein, *F. solani* with 83 nkat/ml of protein, and *F. proliferatum* with 81 nkat/mg of protein (Boudabbous *et al*., 2016; Gao *et al*., 2012; Ravalason *et al*., 2012; Su *et al*., 2006; Su *et al*., 2009; Xiros *et al*., 2008; Zhao *et al*., 2013). Our results from the baseline assay indicate β-glucosidase activity was present in the intracellular protein of *F. proliferatum* with an activity of 115.7 nkat/mg of protein towards 4–nitrophenyl–β–D–glucopyranoside (**Fig. 14**). Future studies should focus on both the intracellular and extracellular protein fractions for a holistic β-glucosidase enzyme analysis of *F. proliferatum*.

Many *Fusarium* species have been applied to the degradation of lignocellulosic biomass for the production of bioethanol (Almeida *et al*., 2014; Behera and Ray, 2016; Indira *et al*., 2016; Olajuyigbe *et al*., 2016; Panagiotou *et al*., 2011; Xiros *et al*., 2011). Members of *Fusarium* are known proficient producers of cellulases and fully capable of utilizing cellulose as the sole carbon substrate (Gupta and Verma, 2015). *Fusarium* species have been grown and analyzed on cellulosic substrates such as corn cob, corn starch, straw, wheat bran and brewers' grain (Ali *et al*., 2016; Almeida *et al*., 2014; Anasontzis *et al*., 2011; Baeyens *et al*., 2015; Vohra *et*

al., 2014; Xiros *et al*., 2011; Xu *et al*., 2015). The use of *F. oxysporum* for the production of bioethanol using brewers' grain and corn cob as the carbon source yielded 65 g ethanol/kg of brewers' grain (Xiros *et al*., 2008). Similarly, using rice straw as a carbon source (1-10 % w/v), *F. oxysporum* was able to produce 0.125 g ethanol/g of rice straw under high ionic liquids (Xu *et al*., 2015). Hypothetically, if similar yields of ethanol production were obtained using the total amount of cigarette filters littered annually, a maximum yield of 6 million kg of ethanol would be obtained. These studies on *F. oxysporum*, suggest promising results for *Fusarium* species in fungal-based biorefineries for the production of bioethanol (Xiros *et al*., 2008; Xu *et al*., 2015).

Future prospects

Although, the comparison for *F. proliferatum* of baseline activity and enhanced cellulolytic expression did not occur, the experimental design was devised. This future experiment would be inoculations of *F. proliferatum* for the comparison of baseline enzyme activity grown in LB from glycerol stock, enzyme activity grown in CMC from glycerol stock, and enhanced enzyme activity grown in CMC from enhanced cellulolytic expression. Both the intracellular and extracellular protein fractions should be investigated using the same pNP-linked substrates used in this study. If improved enzyme activities for acetyl esterases, β-glucosidase, and cellulases are obtained when grown on CMC, direct extrapolation for cellulose acetate degradation may be accurate. Using the enhanced cellulolytic expression of *F. proliferatum* for the inoculation of CMC would provide a better idea of the genetic adaptability and the potential application for improved cellulose acetate degradation.

Future studies should not exclude the three *M. circinelloides* isolates harvested from the cigarette bin. The living synergistic microbiome of the cigarette bin enabled the microorganisms to thrive in this ecological niche. Co-inoculation studies should be investigated in order to try mimic the synergistic microbiome of the cigarette bin. *Mucor circinelloides* have been reported to produce a complete cellulolytic cocktail enabling the utility of cellulose and cellobiose as the sole carbon substrate (Alves *et al*., 2002; Huang *et al*., 2014; Saha and Bothast, 1999; Saha, 2004; Shimonaka *et al*., 2006; Takano *et al*., 2012). Additionally, *Mucor circinelloides* produces mycelia-bound lipases, of which over 20 % of the total biomass can be lipid derivatives suggesting potential acetyl esterase activity (Andrade *et al*., 2014; Carvalho *et al*., 2015; Magdum *et al*., 2015; Szczęsna-Antczak *et al*., 2018; Vicente *et al*., 2009). Recently, the utilization of *Mucor circinelloides* for the remediation of polyphosphates and heavy metals has proven highly successful (Cui *et al*., 2017; Zhang *et al*., 2017). In this regard, co-inoculations of I1, I2, I3, and I4, could possibly work synergistically in such a manner that each has a uniquely developed function to assist in the degradation of cigarette filters. Expanding on future experiments, a pilot scale study for the analysis of CO_2 emissions and bioethanol production using the fungal isolates in an anaerobic digestor using cigarette filter waste as the carbon source would provide preliminary results for the potential scale up of a fungal-based biorefinery focused on the remediation of cigarette filter pollution.

References

Abdalla, M., Al-Rokibah, A., Moretti, A. and Mulè, G. (2000) Pathogenicity of toxigenic *Fusarium proliferatum* from date palm in Saudi Arabia. *Plant Disease*, 84(3), 321-324.

Abdel-Azeem, M., Darwish, A., Nafady, N. and Ibrahim, N. (2019) *Fusarium*: biodiversity, ecological significances, and industrial applications. In: Yadav A., Mishra S., Singh S., Gupta A. (eds) Recent advancement in white biotechnology through Fungi. *Fungal Biology*. Springer, Cham.

Abrusci, C., Marquina, D., Santos, A., Del Amo, A., Corrales, T. and Catalina, F. (2009) Biodeterioration of cinematographic cellulose triacetate by *Sphingomonas paucimobilis* using indirect impedance and chemiluminescence techniques. *International Biodeterioration & Biodegradation*, 63(6), 759-764.

Abu-Danso, E., Bagheri, A. and Bhatnagar, A. (2019) Facile functionalization of cellulose from discarded cigarette butts for the removal of diclofenac from water. *Carbohydrate Polymers*, 219, 46-55.

Addamo, A., Larroche, P. and Hanke, G. (2017) Top marine beach litter items in Europe: a review and synthesis based on beach litter data. European Commission, *JRC Technical Reports*, EUR 29249 EN, Luxembourg.

Ainsworth, G. (1965) Historical introduction to Mycology. New York: Academic Press, 3-20.

Akhtar, N. and Mannan, M. (2020) Mycoremediation: Expunging environmental pollutants. *Biotechnology Reports*, 26, e00452.

Alharbi, O., Basheer, A., Khattab, R. and Ali, I. (2018) Health and environmental effects of persistent organic pollutants. *Journal of Molecular Liquids*, 263, 442-453.

Ali, S., Nugent, B., Mullins, E. and Doohan, F. (2016) Fungal-mediated consolidated bioprocessing: the potential of *Fusarium oxysporum* for the lignocellulosic ethanol industry. *AMB Express*, 6(1), 13.

Almeida, M., Guimarães, V., Falkoski, D., Paes, G., Ribeiro, J., Visser, E., Alfenas, R., Pereira, O. and de Rezende, S. (2014) Optimization of endoglucanase and xylanase activities from *Fusarium verticillioides* for simultaneous saccharification and

fermentation of sugarcane bagasse. *Applied Biochemistry and Biotechnology*, 172(3), 1332-1346.

Alves, M., Campos-Takaki, G., Porto, A. and Milanez, A. (2002) Screening of *Mucor* spp. for the production of amylase, lipase, polygalacturonase and protease. *Brazilian Journal of Microbiology*, 33(4).

American Chemical Society. 2020. Methods for microplastics, nanoplastics and plastic monomer detection and reporting in human tissues - American Chemical Society. [online] Available at: <https://www.acs.org/content/acs/en/pressroom/newsreleases/2020/august/micro-and-nanoplastics-detectable-in-human-tissues.html> [Accessed 28 August 2020].

Amouri, B. and Gargouri, A. (2006) Characterization of a novel β-glucosidase from a *Stachybotrys* strain. *Biochemical Engineering Journal*, 32, 191-197.

Anasontzis, G., Zerva, A., Stathopoulou, P., Haralampidis, K., Diallinas, G., Karagouni, A. and Hatzinikolaou, D. (2011) Homologous overexpression of xylanase in *Fusarium oxysporum* increases ethanol productivity during consolidated bioprocessing (CBP) of lignocellulosics. *Journal of Biotechnology*, 152(1-2), 16-23.

Anderson, C. and Juday, G. (2016) Mycoremediation of petroleum: a literature review. *Journal of Environmental Science and Engineering A*, 5, 397-405.

Anderson, J., Byrne, T., Woelfel, K., Meany, J., Spyridis, G. and Pocker, Y. (1994) The hydrolysis of p-nitrophenyl acetate: a versatile reaction to study enzyme kinetics. *Journal of Chemical Education*, 71, 715.

Andrade, G., Carvalho, A., Romero, C., Oliveira, P. and de Castro, H. (2014) *Mucor circinelloides* whole-cells as a biocatalyst for the production of ethyl esters based on babassu oil. *Bioprocess and Biosystems Engineering*, 37(12), 2539-2548.

Andrady, A. (2011) Microplastics in the marine environment. *Marine Pollution Bulletin*, 62(8), 1596-1605.

Anwar, Z., Gulfraz, M. and Irshad, M. (2014) Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review. *Journal of Radiation Research and Applied Sciences,* 7, 163-173.

Araújo, M. and Costa, M. (2019a) A critical review of the issue of cigarette butt pollution in coastal environments. *Environmental Research*, 172, 137-149.

Araújo, M. and Costa, M. (2019b) From plant to waste: the long and diverse impact chain caused by tobacco smoking. *International Journal of Environmental Research and Public Health*, 16(15), 2690.

Ariyawansa, H., Hawksworth, D., Hyde, K., Jones, E., Maharachchikumbura, S., Manamgoda, D., Thambugala, K., Udayanga, D., Camporesi, E., Daranagama, A., Jayawardena, R., Liu, J., McKenzie, E., Phookamsak, R., Senanayake, I., Shivas, R., Tian, Q. and Xu, J. (2014) Epitypification and neotypification: guidelines with appropriate and inappropriate examples. *Fungal Diversity*, 69(1), 57-91.

Atlas, R. (1981) Microbial degradation of petroleum hydrocarbons: an environmental perspective. *Microbiological Reviews*, 45(1), 180-209.

Azubuike, C., Chikere, C. and Okpokwasili, G. (2016) Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects. *World Journal of Microbiology and Biotechnology*, 32(11).

Baayen, R., van den Boogert, P., Bonants, P., Poll, J., Blok, W. and Waalwijk, C. (2000) *Fusarium redolens* f. sp *asparagi*, causal agent of asparagus root rot, crown rot and spear rot. *European Journal of Plant Pathology*, 106(9), 907-912.

Baeyens, J., Kang, Q., Appels, L., Dewil, R., Lv, Y. and Tan, T. (2015) Challenges and opportunities in improving the production of bio-ethanol. *Progress in Energy and Combustion Science*, 47, 60-88.

Balaji, V., Arulazhagan, P. and Ebenezer, P. (2014) Enzymatic bioremediation of polyaromatic hydrocarbons by fungal consortia enriched from petroleum contaminated soil and seeds. *Journal of Environmental Biology*, 35, 521-529.

Baldrian, P. and Valášková, V. (2008) Degradation of cellulose by basidiomycetous fungi. *FEMS Microbiology Reviews*, 32, 501-521.

Barrick, J. and Lenski, R. (2013) Genome dynamics during experimental evolution. *Nature Reviews Genetics*, 14(12), 827-839.

Barrios, N., Kirkpatrick, D., Murciano, A., Stine, K., Van Dyke, R. and Humbert, J. (1990) Successful treatment of disseminated *Fusarium* infection in an immunocompromised child. *Journal of Pediatric Hematology/Oncology*, 12(3), 319-324.

Bashyal, B., Rawat, K., Sharma, S., Kulshreshtha, D., Gopala Krishnan, S., Singh, A., Dubey, H., Solanke, A., Sharma, T. and Aggarwal, R. (2017) Whole genome sequencing of *Fusarium fujikuroi* provides insight into the role of secretory proteins and cell wall degrading enzymes in causing bakanae disease of rice. *Frontiers in Plant Science*, (8).

Bayer, E. (2019) *The Mycelium Revolution Is Upon Us*. [online] Scientific American Blog Network. Available at: <https://blogs.scientificamerican.com/observations/the-mycelium-revolution-is-upon-us/> [Accessed 26 August 2020].

Bayer, E., Belaich, J., Shoham, Y. and Lamed, R. (2004) The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides. *Annual Review of Microbiology*, 58(1), 521-554.

Bayer, E., Chanzy, H., Lamed, R. and Shoham, Y. (1998) Cellulose, cellulases and cellulosomes. *Current Opinion in Structural Biology*, 8(5), 548-557.

Bayer, E., Morag, E. and Lamed, R. (1994) The cellulosome — A treasure-trove for biotechnology. *Trends in Biotechnology*, 12(9), 379-386.

Béguin, P. and Aubert, J. (1994) The biological degradation of cellulose. *FEMS Microbiology Reviews*, 13(1), 25-58.

Behera, S. and Ray, R. (2016) Solid state fermentation for production of microbial cellulases: recent advances and improvement strategies. *International Journal of Biological Macromolecules*, 86, 656-669.

Beliën, T., Van Campenhout, S., Robben, J. and Volckaert, G. (2006) Microbial endoxylanases: effective weapons to breach the plant cell-wall barrier or, rather, triggers of plant defense systems? *Molecular Plant-Microbe Interactions*, 19(10), 1072-1081.

Berbee, M. and Taylor, J. (2001) Fungal molecular evolution: gene trees and geologic time. *Systematics and Evolution*, 229–245.

Berlemont, R. (2017) Distribution and diversity of enzymes for polysaccharide degradation in fungi. *Scientific Reports*, 7(1), 222.

Bernardi-Wenzel, J., Quecine, M., Azevedo, J. and Pamphile, J. (2016) Agrobacterium-mediated transformation of *Fusarium proliferatum*. *Genetics and Molecular Research*, 15(2).

Bessy, O., Lowry, O. and Brock, M. (1946) A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *Journal of Biological Chemistry*, 164, 321-1329.

Bhattacharya, S., Das, A., Mangai., G., Vignesh., K. and Sangeetha., J. (2011) Mycoremediation of Congo red dye by filamentous fungi. *Brazilian Journal of Microbiology*, 42(4), 1526-1536.

Blum, D., Li, X., Chen, H. and Ljungdahl, L. (1999) Characterization of an acetyl xylan esterase from the anaerobic fungus *Orpinomyces* sp. Strain PC-2. *Applied and Environmental Microbiology*, 65, 3990-3995.

Bokulich, N. and Mills, D. (2013) Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. *Applied and Environmental Microbiology*, 79(8), 2519-2526.

Bonanomi, G., Incerti, G., Cesarano, G., Gaglione, S. and Lanzotti, V. (2015) Cigarette butt decomposition and associated chemical changes assessed by 13C CPMAS NMR. *PLOS ONE*, 10: e0117393.

Bonanomi, G., Maisto, G., De Marco, A., Cesarano, G., Zotti, M., Mazzei, P., Libralato, G., Staropoli, A., Siciliano, A. and de Filippis, F. (2020) The fate of cigarette butts in different environments: decay rate, chemical changes and ecotoxicity revealed by a 5-years decomposition experiment. *Environmental Pollution*, 261, 114108.

Booth, D., Gribben, P. and Parkinson, K. (2015) Impact of cigarette butt leachate on tidepool snails. *Marine Pollution Bulletin*, 95(1), 362-364.

Borowik, A., Wyszkowska, J. and Oszust, K. (2017) Functional diversity of fungal communities in soil contaminated with diesel oil. *Frontiers in Microbiology*, 8, 1862.

Bottalico, A. (1998) *Fusarium* diseases of cereals: species complex and related mycotoxins profiles, in Europe. *Journal of Plant Pathology*, 80, 85-103.

Bottalico, A. and Perrone, G. (2002) Toxigenic *Fusarium* species and mycotoxins associated with head blight in small-grain cereals in Europe. *European Journal of Plant Pathology*, 108(7), 611-624.

Boudabbous, M., Saibi, W., Bouallagui, Z., Dardouri, M., Sayadi, S., Belghith, H., Mechichi, T. and Gargouri, A. (2016) Fast activated charcoal prepurification of *Fusarium solani* β-glucosidase for an efficient oleuropein bioconversion. *Preparative Biochemistry & Biotechnology*, 47, 185-191.

Bridge, P., Roberts, P., Spooner, B. and Panchal, G. (2003) On the unreliability of published DNA sequences. *New Phytologist*, 160(1), 43-48.

Bruns, T., White, T. and Taylor, J. (1991) Fungal molecular systematics. *Annual Review of Ecology and Systematics*, 22(1), 525-564.

Buée, M., Reich, M., Murat, C., Morin, E., Nilsson, R., Uroz, S. and Martin, F. (2009) 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. *New Phytologist*, 184(2), 449-456.

Burgess, L. and Summerell, B. (1992) Mycogeography of *Fusarium*: survey of *Fusarium* species in subtropical and semi-arid grassland soils from Queensland, Australia. *Mycological Research*, 96(9), 780-784.

Cabanne, C. and Donèche, B. (2002) Purification and characterization of two isozymes of polygalacturonase from *Botrytis cinerea*. *Microbiological Research*, 157(3), 183-189.

Cai, D., Li, P., Luo, Z., Qin, P., Chen, C., Wang, Y., Wang, Z. and Tan, T. (2016) Effect of dilute alkaline pretreatment on the conversion of different parts of corn stalk to fermentable sugars and its application in acetone–butanol–ethanol fermentation. *Bioresource Technology*, 211, 117-124.

Carvalho, A., Rivaldi, J., Barbosa, J. and de Castro, H. (2015) Biosynthesis, characterization and enzymatic transesterification of single cell oil of *Mucor circinelloides* – A sustainable pathway for biofuel production. *Bioresource Technology*, 181, 47-53.

Cen, Y., Lin, J., Wang, Y., Wang, J., Liu, Z. and Zheng, Y. (2020) The gibberellin producer *Fusarium fujikuroi*: methods and technologies in the current toolkit. *Frontiers in Bioengineering and Biotechnology*, 8, 232.

Chen, H., Wu, H., Yan, B., Zhao, H., Liu, F., Zhang, H., Sheng, Q., Miao, F. and Liang, Z. (2018a) Core microbiome of medicinal plant *Salvia miltiorrhiza* seed: a rich reservoir of beneficial microbes for secondary metabolism? *International Journal of Molecular Sciences*, 19(3), 672.

Chen, M., Qin, Y., Liu, Z., Liu, K., Wang, F. and Qu, Y. (2010) Isolation and characterization of a β-glucosidase from *Penicillium decumbens* and improving hydrolysis of corncob residue by using it as cellulase supplementation. *Enzyme and Microbial Technology*, 46, 444-449.

Chen, S., Li, J., Lin, J., Bao, K., Fan, J., Zhang, R. and He, W. (2018b) High-throughput sequencing fungal community structures in aging tobacco strips from different growing areas and stalk positions. *Tobacco Science & Technology*, 51(4), 12–19.

Cherry, J. and Fidantsef, A. (2003) Directed evolution of industrial enzymes: an update. *Current Opinion in Biotechnology*, 14(4), 438-443.

Chopyk, J., Chattopadhyay, S., Kulkarni, P., Smyth, E., Hittle, L., Paulson, J., Pop, M., Buehler, S., Clark, P., Mongodin, E. and Sapkota, A. (2017) Temporal variations in cigarette tobacco bacterial community composition and tobacco-specific nitrosamine content are influenced by brand and storage conditions. *Frontiers in Microbiology*, 8, 358.

Christakopoulos, P., Koullas, D., Kekos, D., Koukios, E. and Macris, B. (1991) Direct ethanol conversion of pretreated straw by *Fusarium oxysporum*. *Bioresource Technology*, 35(3), 297-300.

Christakopoulos, P., Mamma, D., Kekos, D. and Macris, B. (1999) Enhanced acetyl esterase production from *Fusarium oxysporum*. *World Journal of Microbiology and Biotechnology*, 15(4), 443-446.

Christakopoulos, P., Nerinckx, W., Kekos, D., Macris, B. and Claeyssens, M. (1996) Purification and characterization of two low molecular mass alkaline xylanases from *Fusarium oxysporum* F3. *Journal of Biotechnology*, 51(2), 181-189.

Clarkson, T. (1995) Environmental contaminants in the food chain. *The American Journal of Clinical Nutrition*, 61(3), 682S-686S.

Copley, S. (2000) Evolution of a metabolic pathway for degradation of a toxic xenobiotic: the patchwork approach. *Trends in Biochemical Sciences*, 25(6), 261-265.

Coronado-Ruiz, C., Avendaño, R., Escudero-Leyva, E., Conejo-Barboza, G., Chaverri, P. and Chavarría, M. (2018) Two new cellulolytic fungal species isolated from a 19th-century art collection. *Scientific Reports*, 8(1), 7492.

Cozar, A., Echevarria, F., Gonzalez-Gordillo, J., Irigoien, X., Ubeda, B., Hernandez-Leon, S., Palma, A., Navarro, S., Garcia-de-Lomas, J., Ruiz, A., Fernandez-de-Puelles, M. and Duarte, C. (2014) Plastic debris in the open ocean. *Proceedings of the National Academy of Sciences*, 111(28), 10239-10244.

Cuadros-Orellana, S. (2013) Assessment of fungal diversity in the environment using metagenomics: a decade in review. *Fungal Genomics & Biology*, 3(2), 1-14.

Cui, Z., Zhang, X., Yang, H. and Sun, L. (2017) Bioremediation of heavy metal pollution utilizing composite microbial agent of *Mucor circinelloides*, *Actinomucor* sp. and *Mortierella* sp. *Journal of Environmental Chemical Engineering*, 5(4), 3616-3621.

Dai, Y., Cui, B., Si, J., He, S., Hyde, K., Yuan, H., Liu, X. and Zhou, L. (2015) Dynamics of the worldwide number of fungi with emphasis on fungal diversity in China. *Mycological Progress*, 14(8).

Daroit, D., Simonetti, A., Hertz, P. and Brandelli, A. (2008) Purification and characterization of an extracellular β-Glucosidase from *Monascus purpureus*. *Journal of Microbiology and Biotechnology*, 18(5), 933-941.

de Groot, M., Bundock, P., Hooykaas, P. and Beijersbergen, A. (1998) *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nature Biotechnology*, 16(9), 839-842.

de Souza, C., Lima, D., de Oliveira, R., Gurgel, L. and de A. Santiago, A. (2017) *Mucor indicus* isolated from the semiarid region of Brazil: a first record for South America. *Mycotaxon*, 131(4), 897-906.

Dear, R., Van Kan, J., Pretorius, Z., Hammond-Kosack, K., Di Pietro, A., Spanu, P., Rudd, J., Dickman, M., Kahmann, R., Ellis, J. and Foster, G. (2012) The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology*, 13(4), 414-430.

Deshmukh, R., Khardenavis, A. and Purohit, H. (2016) Diverse metabolic capacities of fungi for bioremediation. *Indian Journal of Microbiology*, 56, 247-264.

Di Giacomo, S., Mazzanti, G. and Di Sotto, A. (2015) Mutagenicity of cigarette butt waste in the bacterial reverse mutation assay: The protective effects of β-caryophyllene and β-caryophyllene oxide. *Environmental Toxicology*, 31(11), 1319-1328.

Dikshit, R. and Tallapragada, P. (2015) Partial purification and characterization of β-glucosidase from *Monascus sanguineus*. *Brazilian Archives of Biology and Technology*. 58, 185-191.

Divne, C., Stahlberg, J., Reinikainen, T., Ruohonen, L., Pettersson, G., Knowles, J., Teeri, T. and Jones, T. (1994) The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei*. *Science*, 265, 524-528.

Dix, N. and Webster, J. (1995) Fungal Ecology. *Springer Netherlands*.

Dobaradaran, S., Schmidt, T., Nabipour, I., Ostovar, A., Raeisi, A., Saeedi, R., Khorsand, M., Khajeahmadi, N. and Keshtkar, M. (2018) Cigarette butts abundance and association of mercury and lead along the Persian Gulf beach: an initial investigation. *Environmental Science and Pollution Research*, 25(6), 5465-5473.

Donaghy, J. and McKay, A. (1992) Extracellular carboxylesterase activity of *Fusarium graminearum*. *Applied Microbiology and Biotechnology*, 37, 742-744.

Eaton, T., Falkinham, J. and von Reyn, C. (1995) Recovery of *Mycobacterium avium* from cigarettes. *Journal of Clinical Microbiology*, 33(10), 2757-2758.

El Hadri, H., Lisa, J., Gigault, J., Reynaud, S. and Grassl, B. (2020) Fate of nanoplastics in the environment: implication of the cigarette butts. *Environmental Pollution*, 268, 115170.

Elleuche, S. and Poggeler, S. (2009) Carbonic anhydrases in fungi. *Microbiology*, 156, 23-29.

Erickson, B., Nelson, J. and Winters, P. (2012) Perspective on opportunities in industrial biotechnology in renewable chemicals. *Biotechnology Journal*, 7(2), 176-185.

Fabiano, M., Danovaro, R., Magi, E. and Mazzucotelli, A. (1994) Effects of heavy metals on benthic bacteria in coastal marine sediments: a field result. *Marine Pollution Bulletin*, 28(1), 18-23.

Fantroussi, S. and Agathos, S. (2005) Is bioaugmentation a feasible strategy for pollutant removal and site remediation? *Current Opinion in Microbiology*, 8, 268-275.

Fernández-Martín, R., Cerdá-Olmedo, E. and Avalos, J. (2000) Homologous recombination and allele replacement in transformants of *Fusarium fujikuroi*. *Molecular and General Genetics MGG*, 263(5), 838-845.

Ford, T. (1994) Pollutant effects on the microbial ecosystem. *Environmental Health Perspectives*, 102(12), 45-48.

Gao, Z., van Hop, D., Yen, L., Ando, K., Hiyamuta, S. and Kondo, R. (2012) The production of β-glucosidases by *Fusarium proliferatum* NBRC109045 isolated from Vietnamese forest. *AMB Express*, 2(49).

García-Martínez, J., Brunk, M., Avalos, J. and Terpitz, U. (2015) The CarO rhodopsin of the fungus *Fusarium fujikuroi* is a light-driven proton pump that retards spore germination. *Scientific Reports*, 5(1).

Gardes, M. and Bruns, T. (1993) ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology*, 2(2), 113-118.

Geiser, D., Aoki, T., Bacon, C., Baker, S., Bhattacharyya, M., Brandt, M., Brown, D., Burgess, L., Chulze, S., Coleman, J., Correll, J., Covert, S., Crous, P., Cuomo, C., De Hoog, G., Di Pietro, A., Elmer, W., Epstein, L., Frandsen, R., Freeman, S., Gagkaeva, T., Glenn, A., Gordon, T., Gregory, N., Hammond-Kosack, K., Hanson, L., Jímenez-Gasco, M., Kang, S., Kistler, H., Kuldau, G., Leslie, J., Logrieco, A., Lu, G., Lysøe, E., Ma, L., McCormick, S., Migheli, Q., Moretti, A., Munaut, F., O'Donnell, K., Pfenning, L., Ploetz, R., Proctor, R., Rehner, S., Robert, V., Rooney, A., bin Salleh, B., Scandiani, M., Scauflaire, J., Short, D., Steenkamp, E., Suga, H., Summerell, B., Sutton, D., Thrane, U., Trail, F., Van Diepeningen, A., VanEtten, H., Viljoen, A., Waalwijk, C., Ward, T., Wingfield, M., Xu, J., Yang, X., Yli-Mattila, T. and Zhang, N. (2013) One Fungus, One Name: defining the genus *Fusarium* in a scientifically robust way that preserves longstanding use. *Phytopathology*, 103(5), 400-408.

Ghatora, S., Chadha, B., Saini, H., Bhat, M. and Faulds, C. (2006) Diversity of plant cell wall esterases in thermophilic and thermotolerant fungi. *Journal of Biotechnology*, 125, 434-445.

Gillespie, I. and Philp, J. (2013) Bioremediation, an environmental remediation technology for the bioeconomy. *Trends in Biotechnology*, 31, 329-332.

Glass, N. and Donaldson, G. (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Applied and Environmental Microbiology*, 61(4), 1323-1330.

Glenn, A. (2007) Mycotoxigenic *Fusarium* species in animal feed. *Animal Feed Science and Technology*, 137(3-4), 213-240.

Gohel, H., Contractor, C., Ghosh, S. and Braganza, V. (2014) A comparative study of various staining techniques for determination of extracellular cellulase activity on Carboxy Methyl Cellulose (CMC) agar plates. *International Journal of Current Microbiology and Applied Sciences*, 3(5), 261-266.

Golias, H., Dumsday, G., Stanley, G. and Pamment, N. (2000) *Biotechnology Letters*, 22(7), 617-621.

Gopinath, S., Hilda, A. and Anbu, P. (2005) Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments. *Mycoscience*, 46, 119-126.

Goswami, R., and Kistler, H. (2004) Heading for disaster: *Fusarium graminearum* on cereal crops. *Molecular Plant Pathology*, 5(6), 515-525.

Gräfenhan, T., Schroers, H., Nirenberg, H. and Seifert, K. (2011) An overview of the taxonomy, phylogeny, and typification of nectriaceous fungi in *Cosmospora, Acremonium, Fusarium, Stilbella*, and *Volutella*. *Studies in Mycology*, 68, 79-113.

Green, D., Boots, B., Da Silva Carvalho, J. and Starkey, T. (2019) Cigarette butts have adverse effects on initial growth of perennial ryegrass (gramineae: *Lolium perenne* L.) and white clover (leguminosae: *Trifolium repens* L.). *Ecotoxicology and Environmental Safety*, 182, 109418.

Green, D., Kregting, L. and Boots, B. (2020) Smoked cigarette butt leachate impacts survival and behaviour of freshwater invertebrates. *Environmental Pollution*, 266, 115286.

Green, R., Putschew, A. and Nehls, T. (2014) Littered cigarette butts as a source of nicotine in urban waters. *Journal of Hydrology*, 519, 3466-3474.

Groenewald, M., Vu, D., de Vries, M., Gehrmann, T., Stielow, B., Eberhardt, U., Al-Hatmi, A., Groenewald, J., Cardinali, G., Houbraken, J., Boekhout, T., Crous, P., Robert, V. and Verkley, G. (2019) Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholds for fungal species and higher taxon delimitation. *Studies in Mycology*, 92, 135-154.

Gupta, A. and Verma, J. (2015) Sustainable Bio-Ethanol Production From Agro-Residues: A Review. *Renewable and Sustainable Energy Reviews*, 41, 550-567.

Guzmán, G., Sandberg, T., LaCroix, R., Nyerges, Á., Papp, H., de Raad, M., King, Z., Hefner, Y., Northen, T., Notebaart, R., Pál, C., Palsson, B., Papp, B. and Feist, A. (2019) Enzyme promiscuity shapes adaptation to novel growth substrates. *Molecular Systems Biology*, 15(4).

Hamzah, Y. and Umar, L. (2017) Preparation of creating active carbon from cigarette filter waste using microwave-induced KOH activation. *Journal of Physics: Conference Series*, 853, 012027.

Hankin, L. and Anagnostakis, S. (1975) The use of solid media for detection of enzyme production by Fungi. *Mycologia*, 67, 597.

Harris, B. (2011) The intractable cigarette 'filter problem'. *Tobacco Control*, 20, 10-16.

Haske-Cornelius, O., Pellis, A., Tegl, G., Wurz, S., Saake, B., Ludwig, R., Sebastian, A., Nyanhongo, G. and Guebitz, G. (2017) Enzymatic systems for cellulose acetate degradation. *Catalysts*, 7, 287.

Hawksworth, D. (2004) Fungal diversity and its implications for genetic resource collections. *Studies in Mycology*, 50(1), 9–17.

Hawthorne, B., Rees-George, J. and Crowhurst, R. (2001) Induction of cutinolytic esterase activity during saprophytic growth of cucurbit pathogens, *Fusarium solani* f. sp. *cucurbitaeraces* one and two (*Nectria haematococca* MPI and MPV, respectively). *FEMS Microbiology Letters*, 194(2), 135-141.

Herbrecht, R., Kessler, R., Kravanja, C., Meyer, M., Waller, J. and Letscher-Bru, V. (2004) Successful treatment of *Fusarium proliferatum* pneumonia with posaconazole in a lung transplant recipient. *The Journal of Heart and Lung Transplantation*, 23(12), 1451-1454.

Hermet, A., Méheust, D., Mounier, J., Barbier, G. and Jany, J. (2012) Molecular systematics in the genus *Mucor* with special regards to species encountered in cheese. *Fungal Biology*, 116(6), 692-705.

Hibbett, D., Binder, M., Bischoff, J., Blackwell, M., Cannon, P., Eriksson, O., Huhndorf, S., James, T., Kirk, P., Lücking, R., Thorsten Lumbsch, H., Lutzoni, F., Matheny, P., McLaughlin, D., Powell, M., Redhead, S., Schoch, C., Spatafora, J., Stalpers, J., Vilgalys, R., Aime, M., Aptroot, A., Bauer, R., Begerow, D., Benny, G., Castlebury, L., Crous, P., Dai, Y., Gams, W., Geiser, D., Griffith, G., Gueidan, C., Hawksworth, D., Hestmark, G., Hosaka, K., Humber, R., Hyde, K., Ironside, J., Kõljalg, U., Kurtzman, C., Larsson, K., Lichtwardt, R., Longcore, J., Miądlikowska, J., Miller, A., Moncalvo, J., Mozley-Standridge, S., Oberwinkler, F., Parmasto, E., Reeb, V., Rogers, J., Roux, C., Ryvarden, L., Sampaio, J., Schüßler, A., Sugiyama, J., Thorn, R., Tibell, L., Untereiner, W., Walker, C., Wang, Z., Weir, A., Weiss, M., White, M., Winka, K., Yao, Y. and Zhang, N. (2007) A higher-level phylogenetic classification of the Fungi. *Mycological Research*, 111(5), 509-547.

Hibbett, D., Ohman, A., Glotzer, D., Nuhn, M., Kirk, P. and Nilsson, R. (2011) Progress in molecular and morphological taxon discovery in Fungi and options for formal classification of environmental sequences. *Fungal Biology Reviews*, 25(1), 38-47.

Ho, L., Martin, D. and Lindemann, W. (1983) Inability of micro-organisms to degrade cellulose acetate reverse-osmosis membranes. *Applied and Environmental Microbiology*, 45(2), 418-427.

Hoffmann, D. and Hoffmann, I. (1997) The changing cigarette, 1950-1995. *Journal of Toxicology and Environmental Health*, 50(4), 307-364.

Hoffmann, K., Telle, S., Walther, G., Eckart, M., Kirchmair, M., Prillinger, H., Prazenica, A., Newcombe, G., Dölz, F., Papp, T., Vágvölgyi, C., deHoog, G., Olsson, L. and Voigt, K. (2008) Diversity, genotypic identification, ultrastructural and phylogenetic characterization of zygomycetes from different ecological habitats and climatic regions: Limitations and utility of nuclear ribosomal DNA barcode markers. *Current Advances in Molecular Mycology*, 263-312.

Hon, D. (1994) Cellulose: a random walk along its historical path. *Cellulose*, 1, 1-25.

Hosokawa, R., Nagai, M., Morikawa, M. and Okuyama, H. (2009) Autochthonous bioaugmentation and its possible application to oil spills. *World Journal of Microbiology and Biotechnology*, 25, 1519-1528.

Hosono, K., Kanazawa, A., Mori, H. and Endo, T. (2007) Photodegradation of cellulose acetate film in the presence of benzophenone as a photosensitizer. *Journal of Applied Polymer Science*, 105(6), 3235-3239.

Huang, J., Yang, J., Duan, Y., Gu, W., Gong, X., Zhe, W., Su, C. and Zhang, K. (2010) Bacterial diversities on unaged and aging flue-cured tobacco leaves estimated by 16S rRNA sequence analysis. *Applied Microbiology and Biotechnology*, 88(2), 553-562.

Huang, R., Hippauf, F., Rohrbeck, D., Haustein, M., Wenke, K., Feike, J., Sorrelle, N., Piechulla, B. and Barkman, T. (2012) Enzyme functional evolution through improved catalysis of ancestrally nonpreferred substrates. *Proceedings of the National Academy of Sciences*, 109(8), 2966-2971.

Huang, Y., Busk, P., Grell, M., Zhao, H. and Lange, L. (2014) Identification of a β-glucosidase from the *Mucor circinelloides* genome by peptide pattern recognition. *Enzyme and Microbial Technology*, 67, 47-52.

Hyde, K., Xu, J., Rapior, S., Jeewon, R., Lumyong, S., Niego, A., Abeywickrama, P., Aluthmuhandiram, J., Brahamanage, R., Brooks, S., Chaiyasen, A., Chethana, K., Chomnunti, P., Chepkirui, C., Chuankid, B., de Silva, N., Doilom, M., Faulds, C., Gentekaki, E., Gopalan, V., Kakumyan, P., Harishchandra, D., Hemachandran, H., Hongsanan, S., Karunarathna, A., Karunarathna, S., Khan, S., Kumla, J., Jayawardena, R., Liu, J., Liu, N., Luangharn, T., Macabeo, A., Marasinghe, D., Meeks, D., Mortimer, P., Mueller, P., Nadir, S., Nataraja, K., Nontachaiyapoom, S., O'Brien, M., Penkhrue, W., Phukhamsakda, C., Ramanan, U., Rathnayaka, A., Sadaba, R., Sandargo, B., Samarakoon, B., Tennakoon, D., Siva, R., Sriprom, W., Suryanarayanan, T., Sujarit, K., Suwannarach, N., Suwunwong, T., Thongbai, B., Thongklang, N., Wei, D., Wijesinghe, S., Winiski, J., Yan, J., Yasanthika, E. and Stadler, M. (2019) The amazing potential of fungi: 50 ways we can exploit fungi industrially. *Fungal Diversity*, 97(1), 1-136.

Indira, D., Sharmila, D., Balasubramanian, P., Thirugnanam, A. and Jayabalan, R. (2016) Utilization of sea water based media for the production and characterization of cellulase

by *Fusarium subglutinans* MTCC 11891. *Biocatalysis and Agricultural Biotechnology*, 7, 187-192.

International Barcode of Life. DNA Barcoding - International Barcode Of Life. [online] Available at: <https://ibol.org/about/dna-barcoding/> [Accessed 28 August 2020].

Islam, M., Nizam, S. and Verma, P. (2012) A highly efficient *Agrobacterium* mediated transformation system for chickpea wilt pathogen *Fusarium oxysporum* f. sp. *ciceri* using DsRed-Express to follow root colonisation. *Microbiological Research*, 167(6), 332-338.

Itävaara, M., Siika-aho, M. and Viikari, L. (1999) Enzymatic degradation of cellulose-based materials. *Journal of Polymers and the Environment*, 7(2), 67-73.

Jacobs-Venter, A., Laraba, I., Geiser, D., Busman, M., Vaughan, M., Proctor, R., McCormick, S. and O'Donnell, K. (2018) Molecular systematics of two sister clades, the *Fusarium concolor* and *F. babinda* species complexes, and the discovery of a novel microcycle macroconidium–producing species from South Africa. *Mycologia*, 110(6), 1189-1204.

James, T., Kauff, F., Schoch, C., Matheny, P., Hofstetter, V., Cox, C., Celio, G., Gueidan, C., Fraker, E., Miadlikowska, J., Lumbsch, H., Rauhut, A., Reeb, V., Arnold, A., Amtoft, A., Stajich, J., Hosaka, K., Sung, G., Johnson, D., O'Rourke, B., Crockett, M., Binder, M., Curtis, J., Slot, J., Wang, Z., Wilson, A., Schüßler, A., Longcore, J., O'Donnell, K., Mozley-Standridge, S., Porter, D., Letcher, P., Powell, M., Taylor, J., White, M., Griffith, G., Davies, D., Humber, R., Morton, J., Sugiyama, J., Rossman, A., Rogers, J., Pfister, D., Hewitt, D., Hansen, K., Hambleton, S., Shoemaker, R., Kohlmeyer, J., Volkmann-Kohlmeyer, B., Spotts, R., Serdani, M., Crous, P., Hughes, K., Matsuura, K., Langer, E., Langer, G., Untereiner, W., Lücking, R., Büdel, B., Geiser, D., Aptroot, A., Diederich, P., Schmitt, I., Schultz, M., Yahr, R., Hibbett, D., Lutzoni, F., McLaughlin, D., Spatafora, J. and Vilgalys, R. (2006) Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature*, 443(7113), 818-822.

Jensen, R. (1976) Enzyme recruitment in evolution of new function. *Annual Review of Microbiology*, 30(1), 409-425.

Jeong, H., Lee, S., Choi, G., Lee, T. and Yun, S. (2013) Draft genome sequence of *Fusarium fujikuroi* B14, the causal agent of the bakanae disease of rice. *Genome Announcements*, 1(1).

Jeschke, N., Nelson, P. and Marasas, W. (1990) *Fusarium* species isolated from soil samples collected at different altitudes in the Transkei, Southern Africa. *Mycologia*, 82(6), 727-733.

Johnsen, H. and Krause, K. (2014) Cellulase activity screening using pure carboxymethylcellulose: application to soluble cellulolytic samples and to plant tissue prints. *International Journal of Molecular Sciences*, 15(1), 830-838.

Joo, A., Jeya, M., Lee, K., Sim, W., Kim, J., Kim, I., Kim, Y., Oh, D., Gunasekaran, P. and Lee, J. (2009) Purification and characterization of a β-1,4-glucosidase from a newly isolated strain of *Fomitopsis pinicola*. *Applied Microbiology and Biotechnology*, 83, 285-294.

Jortner, J. (1959) Photochemistry of cellulose acetate. *Journal of Polymer Science*, 37(131), 199-214.

Kandeler, E., Tscherko, D., Bruce, K., Stemmer, M., Hobbs, P., Bardgett, R. and Amelung, W. (2000) Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil. *Biology and Fertility of Soils*, 32(5), 390-400.

Kapahi, M. and Sachdeva, S. (2017) Mycoremediation potential of *Pleurotus* species for heavy metals: a review. *Bioresources and Bioprocessing*, 4(1).

Karnchanatat, A., Petsom, A., Sangvanich, P., Piaphukiew, J., Whalley, A., Reynolds, C. and Sihanonth, P, (2007) Purification and biochemical characterization of an extracellular Î²-glucosidase from the wood-decaying fungus *Daldinia eschscholzii*. *FEMS Microbiology Letters*, 270, 162-170.

Kataržytė, M., Balčiūnas, A., Haseler, M., Sabaliauskaitė, V., Lauciūtė, L., Stepanova, K., Nazzari, C. and Schernewski, G. (2020) Cigarette butts on Baltic Sea beaches: monitoring, pollution and mitigation measures. *Marine Pollution Bulletin*, 156, 111248.

Kaur, J., Chadha, B., Kumar, B. and Saini, H. (2007) Purification and characterization of two endoglucanases from *Melanocarpus* sp. MTCC 3922. *Bioresource Technology*, 98, 74-81.

Khan, I., Ali, M., Aftab, M., Shakir, S., Qayyum, S., Haleem, K. and Tauseef, I. (2019) Mycoremediation: a treatment for heavy metal-polluted soil using indigenous metallotolerant fungi. *Environmental Monitoring and Assessment*, 191, 622.

Khersonsky, O. and Tawfik, D. (2010) Enzyme promiscuity: a mechanistic and evolutionary perspective. *Annual Review of Biochemistry*, 79(1), 471-505.

Kim, Y., Seo, H., Min, J., Kim, Y., Ban, Y., Han, K., Park, J., Bae, K., Gu, M. and Lee, J. (2007) Enhanced degradation and toxicity reduction of dihexyl phthalate by *Fusarium oxysporum* f. sp. *pisi cutinase*. *Journal of Applied Microbiology*, 102(1), 221-228.

King, E. and Nicholson, T. (1939) The preparation of phenyl phosphoric esters. *Biochemical Journal*, 33(8), 1182–1184.

Knogge, W. (1996) Fungal infection of plants. *The Plant Cell*, 8(10), 1711-1722.

Knott, B., Crowley, M., Himmel, M., Ståhlberg, J. and Beckham, G. (2014) Carbohydrate–protein interactions that drive processive polysaccharide translocation in enzymes revealed from a computational study of cellobiohydrolase processivity. *Journal of the American Chemical Society*, 136, 8810-8819.

Kolattukudy, P. (2005) Cutin from plants. *Biopolymers Online*.

Kolattukudy, P., Rogers, L., Li, D., Hwang, C. and Flaishman, M. (1995) Surface signalling in pathogenesis. *Proceedings of the National Academy of Sciences*, 92, 4080-4087.

Kõljalg, U., Nilsson, R., Abarenkov, K., Tedersoo, L., Taylor, A., Bahram, M., Bates, S., Bruns, T., Bengtsson-Palme, J., Callaghan, T., Douglas, B., Drenkhan, T., Eberhardt, U., Dueñas, M., Grebenc, T., Griffith, G., Hartmann, M., Kirk, P., Kohout, P., Larsson, E., Lindahl, B., Lücking, R., Martín, M., Matheny, P., Nguyen, N., Niskanen, T., Oja, J., Peay, K., Peintner, U., Peterson, M., Põldmaa, K., Saag, L., Saar, I., Schüßler, A., Scott, J., Senés, C., Smith, M., Suija, A., Taylor, D., Telleria, M., Weiss, M. and Larsson, K. (2013) Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology*, 22(21), 5271-5277.

Kraemer, S., Ramachandran, A. and Perron, G. (2019) Antibiotic pollution in the environment: from microbial ecology to public policy. *Microorganisms*, 7(6), 180.

Kuhad, R., Gupta, R. and Singh, A. (2011) Microbial cellulases and their industrial applications. *Enzyme Research*, 2011, 1-10.

Kuhad, R., Singh, A. and Eriksson, K. (1997) Micro-organisms and enzymes involved in the degradation of plant fiber cell walls. *Advances in Biochemical Engineering Biotechnology*, 57, 45-125.

Kulik, T., Pszczółkowska, A., Fordoński, G. and Olszewski, J. (2007) PCR approach based on the *esyn1* gene for the detection of potential enniatin-producing *Fusarium* species. *International Journal of Food Microbiology*, 116(3), 319-324.

Kungskulniti, N., Charoenca, N., Hamann, S., Pitayarangsarit, S. and Mock, J. (2018) Cigarette waste in popular beaches in Thailand: high densities that demand environmental action. *International Journal of Environmental Research and Public Health*, 15(4), 630.

Kurmus, H. and Mohajerani, A. (2020) The toxicity and valorization options of cigarette butts. *Waste Management*, 104, 104-118.

Kvas, M., Steenkamp, E., Wingfield, B., Marasas, W. and Wingfield, M. (2008) Diversity of *Fusarium* species associated with malformed inflorescences of *Syzygium cordatum*. *South African Journal of Botany*, 74(2), 370.

Kwasna, H., Ward, E., and Bateman, G. (2006) Phylogenetic relationships among Zygomycetes from soil based on ITS1/2 rDNA sequences. *Mycological Research*, 110(5), 501-510.

Labbate, M., Seymour, J., Lauro, F. and Brown, M. (2016) Editorial: anthropogenic impacts on the microbial ecology and function of aquatic environments. *Frontiers in Microbiology*, (7).

Laurence, M., Walsh, J., Shuttleworth, L., Robinson, D., Johansen, R., Petrovic, T., Vu, T., Burgess, L., Summerell, B. and Liew, E. (2015) Six novel species of *Fusarium* from natural ecosystems in Australia. *Fungal Diversity*, 77(1), 349-366.

Leppänen, I., Vikman, M., Harlin, A. and Orelma, H. (2019) Enzymatic degradation and pilot-scale composting of cellulose-based films with different chemical structures. *Journal of Polymers and the Environment*, 28(2), 458-470.

Leslie, J. and Summerell, B. (2006) The *Fusarium* laboratory manual. 1st ed. Blackwell Publishing Ltd; Oxford, London.

Li, F., Yuan, G., Liao, T., Li, Q. and Lin, W. (2017) Leaf spot of tobacco caused by *Fusarium proliferatum*. *Journal of General Plant Pathology*, 83(4), 264-267.

Li, G., Hyde, K., Zhao, R., Hongsanan, S., Abdel-Aziz, F., Abdel-Wahab, M., Alvarado, P., Alves-Silva, G., Ammirati, J., Ariyawansa, H., Baghela, A., Bahkali, A., Beug, M., Bhat, D., Bojantchev, D., Boonpratuang, T., Bulgakov, T., Camporesi, E., Boro, M., Ceska, O., Chakraborty, D., Chen, J., Chethana, K., Chomnunti, P., Consiglio, G., Cui, B., Dai, D., Dai, Y., Daranagama, D., Das, K., Dayarathne, M., De Crop, E., De Oliveira, R., de Souza, C., de Souza, J., Dentinger, B., Dissanayake, A., Doilom, M., Drechsler-Santos, E., Ghobad-Nejhad, M., Gilmore, S., Góes-Neto, A., Gorczak, M., Haitjema, C., Hapuarachchi, K., Hashimoto, A., He, M., Henske, J., Hirayama, K., Iribarren, M., Jayasiri, S., Jayawardena, R., Jeon, S., Jerônimo, G., Jesus, A., Jones, E., Kang, J., Karunarathna, S., Kirk, P., Konta, S., Kuhnert, E., Langer, E., Lee, H., Lee, H., Li, W., Li, X., Liimatainen, K., Lima, D., Lin, C., Liu, J., Liu, X., Liu, Z., Luangsa-ard, J., Lücking, R., Lumbsch, H., Lumyong, S., Leaño, E., Marano, A., Matsumura, M., McKenzie, E., Mongkolsamrit, S., Mortimer, P., Nguyen, T., Niskanen, T., Norphanphoun, C., O'Malley, M., Parnmen, S., Pawłowska, J., Perera, R., Phookamsak, R., Phukhamsakda, C., Pires-Zottarelli, C., Raspé, O., Reck, M., Rocha, S., de Santiago, A., Senanayake, I., Setti, L., Shang, Q., Singh, S., Sir, E., Solomon, K., Song, J., Srikitikulchai, P., Stadler, M., Suetrong, S., Takahashi, H., Takahashi, T., Tanaka, K., Tang, L., Thambugala, K., Thanakitpipattana, D., Theodorou, M., Thongbai, B., Thummarukcharoen, T., Tian, Q., Tibpromma, S., Verbeken, A., Vizzini, A., Vlasák, J., Voigt, K., Wanasinghe, D., Wang, Y., Weerakoon, G., Wen, H., Wen, T., Wijayawardene, N., Wongkanoun, S., Wrzosek, M., Xiao, Y., Xu, J., Yan, J., Yang, J., Da Yang, S., Hu, Y., Zhang, J., Zhao, J., Zhou, L., Peršoh, D., Phillips, A. and Maharachchikumbura, S. (2016) Fungal diversity notes 253–366: taxonomic and phylogenetic contributions to fungal taxa. *Fungal Diversity*, 78(1), 1-237.

Li, X., Xu, Z., Yu, J., Huang, H. and Jin, M. (2019) *In situ* pretreatment during distillation improves corn fiber conversion and ethanol yield in the dry mill process. *Green Chemistry*, 21, 1080-1090.

Liu, J., Ma, G., Chen, T., Hou, Y., Yang, S., Zhang, K. and Yang, J. (2015) Nicotine-degrading microorganisms and their potential applications. *Applied Microbiology and Biotechnology*, 99(9), 3775-3785.

Liu, Y. and Hall, B. (2004) Body plan evolution of ascomycetes, as inferred from an RNA polymerase II phylogeny. *Proceedings of the National Academy of Sciences*, 101(13), 4507-4512.

Lladó, S., Covino, S., Solanas, A., Viñas, M., Petruccioli, M. and D'annibale, A. (2013) Comparative assessment of bioremediation approaches to highly recalcitrant PAH degradation in a real industrial polluted soil. *Journal of Hazardous Materials*, 248-249, 407-414.

Lobelle, D. and Cunliffe, M. (2011) Early microbial biofilm formation on marine plastic debris. *Marine Pollution Bulletin*, 62(1), 197-200.

Lombard, L., Sandoval-Denis, M., Lamprecht, S. and Crous, P. (2019a) Epitypification of *Fusarium oxysporum* – clearing the taxonomic chaos. *Persoonia - Molecular Phylogeny and Evolution of Fungi*, 43(1), 1-47.

Lombard, L., van der Merwe, N., Groenewald, J. and Crous, P. (2015) Generic concepts in Nectriaceae. *Studies in Mycology*, 80, 189-245.

Lombard, L., van Doorn, R. and Crous, P. (2019b) Neotypification of *Fusarium chlamydosporum* - a reappraisal of a clinically important species complex. *Fungal Systematics and Evolution*, 4, 183-200.

Lortholary, O., Obenga, G., Biswas, P., Caillot, D., Chachaty, E., Bienvenu, A., Cornet, M., Greene, J., Herbrecht, R., Lacroix, C., Grenouillet, F., Raad, I., Sitbon, K. and Troke, P. (2010) International retrospective analysis of 73 cases of invasive fusariosis treated with Voriconazole. *Antimicrobial Agents and Chemotherapy*, 54(10), 4446-4450.

Luo, Z., Ding, J., Xu, W., Zheng, T. and Zhong, T. (2014) Purification and characterization of an intracellular esterase from a marine *Fusarium* fungal species showing phthalate diesterase activity. *International Biodeterioration & Biodegradation*, 97, 7-12.

Luo, Z., Wu, Y., Chow, R., Luo, J., Gu, J. and Vrijmoed, L. (2012) Purification and characterization of an intracellular esterase from a *Fusarium* species capable of degrading dimethyl terephthalate. *Process Biochemistry*, 47, 687-693.

Lusher, A., McHugh, M. and Thompson, R. (2013) Occurrence of microplastics in the gastrointestinal tract of pelagic and demersal fish from the English Channel. *Marine Pollution Bulletin*, 67(1-2), 94-99.

Lynd, L., Weimer, P., van Zyl, W. and Pretorius, I. (2002) Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews*, 66(3), 506-577.

Lynd, L., Zyl, W., McBride, J. and Laser, M. (2005) Consolidated bioprocessing of cellulosic biomass: an update. *Current Opinion in Biotechnology*, 16(5), 577-583.

Maamar, A., Lucchesi, M., Debaets, S., Nguyen van Long, N., Quemener, M., Coton, E., Bouderbala, M., Burgaud, G. and Matallah-Boutiba, A. (2020) Highlighting the crude oil bioremediation potential of marine fungi isolated from the Port of Oran (Algeria). *Diversity*, 12(5), 196.

Mackay, J., Eriksen, J. and Shafey, O. (2006) The Tobacco Atlas (2nd edn.). The American Cancer Society.

Magdum, S., Minde, G., Adhyapak, U. and Kalyanraman, V. (2015) Competence evaluation of mycodiesel production by oleaginous fungal strains: *Mucor circinelloides* and *Gliocladium roseum*. *International Journal of Energy and Environment*, 6(4), 377-382.

Maldonado-Robledo, G., Rodriguez-Bustamante, E., Sanchez-Contreras, A., Rodriguez-Sanoja, R. and Sanchez, S. (2003) Production of tobacco aroma from lutein: specific role of the microorganisms involved in the process. *Applied Microbiology and Biotechnology*, 62(5-6), 484-488.

Mansouri, N., Etebari, M., Ebrahimi, A., Ebrahimpour, K., Rahimi, B. and Hassanzadeh, A. (2020) Genotoxicity and phytotoxicity comparison of cigarette butt with cigarette ash. *Environmental Science and Pollution Research*, 27, 40383-40391.

Marah, M. and Novotny, T. (2011) Geographic patterns of cigarette butt waste in the urban environment. *Tobacco Control*, 20, 42-44.

Marinello, S., Lolli, F., Gamberini, R. and Rimini, B. (2020) A second life for cigarette butts? A review of recycling solutions. *Journal of Hazardous Materials*, 384, 183-192.

Martin, K. and Rygiewicz, P. (2005) Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. *BMC Microbiology*, 5(1), 28.

Matheny, P., Liu, Y., Ammirati, J. and Hall, B. (2002) Using *RPB1* sequences to improve phylogenetic inference among mushrooms. *American Journal of Botany*, 89(4), 688-698.

McGath, M., Jordan-Mowery, S., Pollei, M., Heslip, S. and Baty, J. (2015) Cellulose acetate lamination: a literature review and survey of paper-based collections in the United States. *Restaurator. International Journal for the Preservation of Library and Archival Material*, 36(4), 333-365.

Meddeb-Mouelhi, F., Moisan, J. and Beauregard, M. (2014) A comparison of plate assay methods for detecting extracellular cellulase and xylanase activity. *Enzyme and Microbial Technology*, 66, 16-19.

Meyer, V., Basenko, E., Benz, J., Braus, G., Caddick, M., Csukai, M., de Vries, R., Endy, D., Frisvad, J., Gunde-Cimerman, N., Haarmann, T., Hadar, Y., Hansen, K., Johnson, R., Keller, N., Kraševec, N., Mortensen, U., Perez, R., Ram, A., Record, E., Ross, P., Shapaval, V., Steiniger, C., van den Brink, H., van Munster, J., Yarden, O. and Wösten, H. (2020) Growing a circular economy with fungal biotechnology: a white paper. *Fungal Biology and Biotechnology*, 7, 5.

Meyer, W. and Gams, W. (2003) Delimitation of *Umbelopsis (Mucorales, Umbelopsidaceae* fam. nov.) based on ITS sequence and RFLP data. *Mycological Research*, 107(3), 339-350.

Micevska, T., Warne, M., Pablo, F. and Patra, R. (2006) Variation in, and causes of, toxicity of cigarette butts to a cladoceran and microtox. *Archives of Environmental Contamination and Toxicology*, 50(2), 205-212.

Migheli, Q., Balmas, V., Harak, H., Sanna, S., Scherm, B., Aoki, T. and O'Donnell, K. (2010) Molecular phylogenetic diversity of dermatologic and other human pathogenic Fusarial isolates from hospitals in Northern and Central Italy. *Journal of Clinical Microbiology*, 48(4), 1076-1084.

Mishra, A. and Malik, A. (2014) Novel fungal consortium for bioremediation of metals and dyes from mixed waste stream. *Bioresource Technology*, 171, 217-226.

Mittal, P., Prasoodanan PK, V., Dhakan, D., Kumar, S. and Sharma, V. (2019) Metagenome of a polluted river reveals a reservoir of metabolic and antibiotic resistance genes. *Environmental Microbiome*, 14, 5.

Molaverdi, M., Karimi, K., Mirmohamadsadeghi, S. and Galbe, M. (2019) High titer ethanol production from rice straw *via* -solid-state simultaneous saccharification and fermentation by *Mucor indicus* at low enzyme loading. *Energy Conversion and Management*, 182, 520-529.

Morin-Sardin, S., Jany, J., Artigaud, S., Pichereau, V., Bernay, B., Coton, E. and Madec, S. (2017) Proteomic analysis of the adaptative response of *Mucor* spp. to cheese environment. *Journal of Proteomics*, 154, 30-39.

Moriwaki, H., Kitajima, S. and Katahira, K. (2009) Waste on the roadside, 'poi-sute' waste: its distribution and elution potential of pollutants into environment. *Waste Management*, 29, 1192-1197.

Moriyoshi, K., Ohmoto, T., Ohe, T. and Sakai, K. (2003) Role of endo-1,4-β-glucanases from *Neisseria sicca* SB in synergistic degradation of cellulose acetate. *Bioscience, Biotechnology, and Biochemistry*, 67, 250-257.

Moriyoshi, K., Yamanaka, H., Ohmoto, T., Ohe, T. and Sakai, K. (2005) Mode of action on deacetylation of acetylated methyl glycoside by cellulose acetate esterase from *Neisseria sicca* SB. *Bioscience, Biotechnology, and Biochemistry*, 69(7), 1292-1299.

Müller, A., Westergaard, K., Christensen, S. and Sørensen, S. (2001) The effect of long-term mercury pollution on the soil microbial community. *FEMS Microbiology Ecology*, 36(1), 11-19.

Nagy, G., Szebenyi, C., Csernetics, Á., Vaz, A., Tóth, E., Vágvölgyi, C. and Papp, T. (2017) Development of a plasmid free CRISPR-Cas9 system for the genetic modification of *Mucor circinelloides*. *Scientific Reports*, 7(1).

Nalim, F., Samuels, G., Wijesundera, R. and Geiser, D. (2011) New species from the *Fusarium solani* species complex derived from perithecia and soil in the Old World tropics. *Mycologia*, 103(6), 1302-1330.

Neish, G., Nelson, P., Toussoun, T. and Clark, R. (1983) *Fusarium*: diseases, biology, and taxonomy. *Mycologia*, 75(1), 190.

Nelson, P., Desjardins, A. and Plattner, R. (1993) Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry, and significance. *Annual Review of Phytopathology*, 31(1), 233-252.

Nelson, P., Dignani, M. and Anaissie, E. (1994) Taxonomy, biology, and clinical aspects of *Fusarium* species. *Clinical Microbiology Reviews*, 7(4), 479-504.

Ng, M., Freeman, M., Fleming, T., Robinson, M., Dwyer-Lindgren, L., Thomson, B., Wollum, A., Sanman, E., Wulf, S., Lopez, A., Murray, C. and Gakidou, E. (2014) Smoking prevalence and cigarette consumption in 187 countries, 1980-2012. *Journal of the American Medical Association*, 311(2), 183-192.

Nicholson, P. (2007) The *Fusarium* Laboratory Manual - by John Leslie. *Plant Pathology*, 56(6), 1037-1037.

Niehaus, E., Kim, H., Münsterkötter, M., Janevska, S., Arndt, B., Kalinina, S., Houterman, P., Ahn, I., Alberti, I., Tonti, S., Kim, D., Sieber, C., Humpf, H., Yun, S., Güldener, U. and Tudzynski, B. (2017) Comparative genomics of geographically distant *Fusarium fujikuroi* isolates revealed two distinct pathotypes correlating with secondary metabolite profiles. *PLOS Pathogens*, 13(10), e1006670.

Nilsson, R., Kristiansson, E., Ryberg, M., Hallenberg, N. and Larsson, K. (2008) Intraspecific ITS variability in the Kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. *Evolutionary Bioinformatics*, 4, EBO.S653.

Nout, M. and Aidoo, K. (2010) Asian fungal fermented food. *Industrial Applications*, 29-58.

Novotny, T. and Slaughter, E. (2014) Tobacco product waste: an environmental approach to reduce tobacco consumption. *Current Environmental Health Reports*, 1, 208-216.

Novotny, T., Lum, K., Smith, E., Wang, V. and Barnes, R. (2009) Cigarettes butts and the case for an environmental policy on hazardous cigarette waste. *International Journal of Environmental Research and Public Health*, 6, 1691-1705.

Nucci, M. and Anaissie, E. (2002) Cutaneous infection by *Fusarium* species in healthy and immunocompromised hosts: implications for diagnosis and management. *Clinical Infectious Diseases*, 35(8), 909-920.

Nyvall, R., Percich, J. and Mirocha, C. (1999) *Fusarium* head blight of cultivated and natural wild rice (*Zizania palustris*) in Minnesota caused by *Fusarium graminearum* and associated *Fusarium* spp. *Plant Disease*, 83(2), 159-164.

O'Donnell, K. and Cigelnik, E. (1997) Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution*, 7(1), 103-116.

O'Donnell, K., Kistler, H., Cigelnik, E. and Ploetz, R. (1998) Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. *Proceedings of the National Academy of Sciences*, 95(5), 2044-2049.

O'Donnell, K., Kistler, H., Tacke, B. and Casper, H. (2000a) Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. *Proceedings of the National Academy of Sciences*, 97(14), 7905-7910.

O'Donnell, K., Lutzoni, F., Ward, T. and Benny, G. (2001) Evolutionary relationships among Mucoralean Fungi (Zygomycota): evidence for family polyphyly on a large scale. *Mycologia*, 93(2), 286.

O'Donnell, K., Nirenberg, H., Aoki, T. and Cigelnik, E. (2000b) A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. *Mycoscience*, 41(1), 61-78.

O'Donnell, K., Rooney, A., Proctor, R., Brown, D., McCormick, S., Ward, T., Frandsen, R., Lysøe, E., Rehner, S., Aoki, T., Robert, V., Crous, P., Groenewald, J., Kang, S. and Geiser, D. (2013) Phylogenetic analyses of *RPB1* and *RPB2* support a middle Cretaceous origin for a clade comprising all agriculturally and medically important fusaria. *Fungal Genetics and Biology*, 52, 20-31.

O'Donnell, K., Sutton, D., Rinaldi, M., Gueidan, C., Crous, P. and Geiser, D. (2009) Novel multilocus sequence typing scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum-equiseti* and *F. chlamydosporum* species complexes within the United States. *Journal of Clinical Microbiology*, 47(12), 3851-3861.

O'Donnell, K., Sutton, D., Rinaldi, M., Sarver, B., Balajee, S., Schroers, H., Summerbell, R., Robert, V., Crous, P., Zhang, N., Aoki, T., Jung, K., Park, J., Lee, Y., Kang, S., Park, B. and Geiser, D. (2010) Internet-accessible DNA sequence database for identifying Fusaria from human and animal infections. *Journal of Clinical Microbiology*, 48(10), 3708-3718.

O'Donnell, K., Ward, T., Aberra, D., Kistler, H., Aoki, T., Orwig, N., Kimura, M., Bjørnstad, Å. and Klemsdal, S. (2008) Multilocus genotyping and molecular phylogenetics resolve a novel head blight pathogen within the *Fusarium graminearum* species complex from Ethiopia. *Fungal Genetics and Biology*, 45(11), 1514-1522.

O'Donnell, K., Ward, T., Robert, V., Crous, P., Geiser, D. and Kang, S. (2015) DNA sequence-based identification of *Fusarium*: current status and future directions. *Phytoparasitica*, 43(5), 583-595.

Obbard, R., Sadri, S., Wong, Y., Khitun, A., Baker, I. and Thompson, R. (2014) Global warming releases microplastic legacy frozen in Arctic Sea ice. *Earth's Future*, 2(6), 315-320.

O'Brien, H., Parrent, J., Jackson, J., Moncalvo, J. and Vilgalys, R. (2005) Fungal community analysis by large-scale sequencing of environmental samples. *Applied and Environmental Microbiology*, 71(9), 5544-5550.

Ocean Conservancy (2018). Building a Clean Swell – 2018 Report.

Olajuyigbe, F., Nlekerem, C. and Ogunyewo, O. (2016) Production and characterization of highly thermostable β-glucosidase during the biodegradation of methyl cellulose by *Fusarium oxysporum*. *Biochemistry Research International*, 2016, 1-8.

Panagiotou, G., Topakas, E., Moukouli, M., Christakopoulos, P. and Olsson, L. (2011) Studying the ability of *Fusarium oxysporum* and recombinant *Saccharomyces cerevisiae* to efficiently cooperate in decomposition and ethanolic fermentation of wheat straw. *Biomass and Bioenergy*, 35(8), 3727-3732.

Park, J., Meriwether, B., Clodfelder, P. and Cunningham, L. (1960) The hydrolysis of p-nitrophenyl acetate catalyzed by 3-phosphoglyceraldehyde dehydrogenase. *The Journal of Biological Chemistry*, 236(1), 136-141.

Parry, D., Jenkinson, P. and McLeod, L. (1995) *Fusarium* ear blight (scab) in small grain cereals - a review. *Plant Pathology*, 44(2), 207-238.

Patel, V., Thomson, G. and Wilson, N. (2012) Cigarette butt littering in city streets: a new methodology for studying and results. *Tobacco Control*, 22(1), 59-62.

Pauly, J., Waight, J. and Paszkiewicz, G., 2008. Tobacco flakes on cigarette filters grow bacteria: a potential health risk to the smoker? 17 Suppl 1, 49-52.

Payne, C., Knott, B., Mayes, H., Hansson, H., Himmel, M., Sandgren, M., Ståhlberg, J. and Beckham, G. (2015) Fungal cellulases. *Chemical Reviews*, 115(3), 1308-1448.

Percival Zhang, Y., Himmel, M. and Mielenz, J. (2006) Outlook for cellulase improvement: screening and selection strategies. *Biotechnology Advances*, 24(5), 452-481.

Pereira, E., Tsang, A., McAllister, T. and Menassa, R. (2013) The production and characterization of a new active lipase from *Acremonium alcalophilum* using a plant bioreactor. *Biotechnology for Biofuels*, 6(111).

Pessôa, M., Paulino, B., Mano, M., Neri-Numa, I., Molina, G. and Pastore, G. (2017) *Fusarium* species—a promising tool box for industrial biotechnology. *Applied Microbiology and Biotechnology*, 101(9), 3493-3511.

Petrikkos, G., Skiada, A., Lortholary, O., Roilides, E., Walsh, T. and Kontoyiannis, D. (2012) Epidemiology and clinical manifestations of Mucormycosis. *Clinical Infectious Diseases*.

Pieters, J. 2018. Identification and biochemical characterisation of an aryl β-glucosidase isolated from a cellulose-acetate rich environment *via* a functional metagenomic approach. [online] Scholar.sun.ac.za. Available at: <https://scholar.sun.ac.za/handle/10019.1/105187> [Accessed 16 August 2020].

Pitt, J. and Hocking, A. (2009) In Fungi and Food Spoilage. Blackie Academic and Professional, London.

Poppendieck, D., Khurshid, S. and Emmerich, S. (2016) Measuring airborne emissions from cigarette butts: literature review and experimental plan. Final Report to U.S Food and Drug Administration Under Interagency Agreement #244-15-9012, 10.6028/NIST.IR.8147.

Potts, J., Clendinning, R. and Ackart, R. (1972) An investigation of the biodegradability of packaging plastics. EPA Study EPA-R2-72-046.

Prabhu, C., Wanjari, S., Gawande, S., Das, S., Labhsetwar, N., Kotwal, S., Puri, A., Satyanarayana, T. and Rayalu, S. (2009) Immobilization of carbonic anhydrase enriched microorganism on biopolymer based materials. *Journal of Molecular Catalysis B: Enzymatic*, 60, 13-21.

Prakash, P., Irinyi, L., Halliday, C., Chen, S., Robert, V. and Meyer, W. (2017) Online databases for taxonomy and identification of pathogenic fungi and proposal for a cloud-based dynamic data network platform. *Journal of Clinical Microbiology*, 55(4), 1011-1024.

Prenafeta-Boldú, F., Kuhn, A., Luykx, D., Anke, H., van Groenestijn, J. and de Bont, J. (2000) Isolation and characterisation of fungi growing on volatile aromatic hydrocarbons as their sole carbon and energy source. *Mycological Research*, 105(4), 477-484.

Prenafeta-Boldú, F., Summerbell, R. and Sybren de Hoog, G. (2006) Fungi growing on aromatic hydrocarbons: biotechnology's unexpected encounter with biohazard? *FEMS Microbiology Reviews*, 30(1), 109-130.

Prieto, A., Möder, M., Rodil, R., Adrian, L. and Marco-Urrea, E. (2011) Degradation of the antibiotics norfloxacin and ciprofloxacin by a white-rot fungus and identification of degradation products. *Bioresource Technology*, 102(23), 10987-10995.

Puls, J., Wilson, S. and Hölter, D. (2010) Degradation of cellulose acetate-based materials: a review. *Journal of Polymers and the Environment,* 19, 152-165.

Qiu, J., Ma, L., Shen, F., Yang, G., Zhang, Y., Deng, S., Zhang, J., Zeng, Y. and Hu, Y. (2017) Pretreating wheat straw by phosphoric acid plus hydrogen peroxide for enzymatic saccharification and ethanol production at high solid loading. *Bioresource Technology*, 238, 174-181.

Rai, B. and Srivastava, A. (1983) Decomposition and competitive colonization of leaf litter by fungi. *Soil Biology and Biochemistry*, 15, 115-117.

Raja, H., Miller, A., Pearce, C. and Oberlies, N. (2017) Fungal identification using molecular tools: a primer for the natural products research community. *Journal of Natural Products*, 80(3), 756-770.

Rambaut, A. (2009). FigTree version 1.3.1 [computer program]

Rath, J., Rubenstein, R., Curry, L., Shank, S. and Cartwright, J. (2012) Cigarette litter: smokers' attitudes and behaviours. *International Journal of Environmental Research and Public Health*, 9, 2189-2203.

Rather, I., Koh, W., Paek, W. and Lim, J. (2017) The sources of chemical contaminants in food and their health implications. *Frontiers in Pharmacology*, 8, 830.

Ravalason, H., Grisel, S., Chevret, D., Favel, A., Berrin, J., Sigoillot, J. and Herpoël-Gimbert, I. (2012) *Fusarium verticillioides* secretome as a source of auxiliary enzymes to enhance saccharification of wheat straw. *Bioresource Technology*, 114, 589-596.

Rehner, S. and Buckley, E. (2005) A Beauveria phylogeny inferred from nuclear ITS and *EF1*- sequences: evidence for cryptic diversification and links to Cordyceps teleomorphs. *Mycologia*, 97(1), 84-98.

Reinthaler, F., Posch, J., Feierl, G., Wüst, G., Haas, D., Ruckenbauer, G., Mascher, F. and Marth, E. (2003) Antibiotic resistance of *E. coli* in sewage and sludge. *Water Research*, 37(8), 1685-1690.

Rhee, S., Liu, X., Wu, L., Chong, S., Wan, X. and Zhou, J. (2004) Detection of genes involved in biodegradation and biotransformation in microbial communities by using 50-mer oligonucleotide microarrays. *Applied and Environmental Microbiology*, 70(7), 4303-4317.

Robertson, R., Thomas, W., Suthar, J. and Brown, D. (2012) Accelerated degradation of cellulose acetate cigarette filters using controlled-release acid catalysis. *Green Chemistry*, 14(8), 2266-2272.

Rocha, A., Di Pietro, A., Ruiz-RolDan, C. and Roncero, M. (2008) *Ctf1*, a transcriptional activator of cutinase and lipase genes in *Fusarium oxysporum* is dispensable for virulence. *Molecular Plant Pathology*, 9, 293-304.

Rodarte-Morales, A., Feijoo, G., Moreira, M. and Lema, J. (2011) Biotransformation of three pharmaceutical active compounds by the fungus *Phanerochaete chrysosporium* in a fed batch stirred reactor under air and oxygen supply. *Biodegradation*, 23(1), 45-156.

Roden, M., Zaoutis, T., Buchanan, W., Knudsen, T., Sarkisova, T., Schaufele, R., Sein, M., Sein, T., Chiou, C., Chu, J., Kontoyiannis, D. and Walsh, T. (2005) Epidemiology and outcome of Zygomycosis: a review of 929 reported cases. *Clinical Infectious Diseases*, 41(5), 634-653.

Rodrigues Reis, C., Bento, H., Carvalho, A., Rajendran, A., Hu, B. and De Castro, H. (2019) Critical applications of *Mucor circinelloides* within a biorefinery context. *Critical Reviews in Biotechnology*, 39(4), 555-570.

Röling, W., van Breukelen, B., Braster, M., Lin, B. and van Verseveld, H. (2001) Relationships between microbial community structure and hydrochemistry in a landfill leachate-polluted aquifer. *Applied and Environmental Microbiology*, 67(10), 4619-4629.

Romberg, M. and Davis, R. (2007) Host range and phylogeny of *Fusarium solani* f. sp. *eumartii* from potato and tomato in California. *Plant Disease*, 91(5), 585-592.

Roncero, M. (2003) *Fusarium* as a model for studying virulence in soilborne plant pathogens. *Physiological and Molecular Plant Pathology*, 62(2), 87-98.

Roncero, M., Jepsen, L., Strøman, P. and van Heeswijck, R. (1989) Characterization of a *leuA* gene and an ARS element from *Mucor circinelloides*. *Gene*, 84(2), 335-343.

Rooney, A., Swezey, J., Wicklow, D. and McAtee, M. (2005) Bacterial species diversity in cigarettes linked to an investigation of severe pneumonitis in U.S. military personnel deployed in operation Iraqi Freedom. *Current Microbiology*, 51(1), 46-52.

Ross, P., Nelson, P., Richard, J., Osweiler, G., Rice, L., Plattner, R. and Wilson, T. (1990) Production of fumonisins by *Fusarium moniliforme* and *Fusarium proliferatum* isolates associated with equine leukoencephalomalacia and a pulmonary edema syndrome in swine. *Applied and Environmental Microbiology*, 56(10), 3225-3226.

Ruiz, M. (1997) Purification and characterization of an acidic endo-β-1,4-xylanase from the tomato vascular pathogen *Fusarium oxysporum* f. sp. *lycopersici*. *FEMS Microbiology Letters*, 148(1), 75-82.

Ruprecht, A., De Marco, C., Saffari, A., Pozzi, P., Mazza, R., Veronese, C., Angellotti, G., Munarini, E., Ogliari, A., Westerdahl, D. *et al*. (2017) Environmental pollution and emission factors of electronic cigarettes, heat-not-burn tobacco products, and conventional cigarettes. *Aerosol Science and Technology*, 51, 674-684.

Saake, B., Horner, S. and Puls, J. (1998) Progress in the enzymatic hydrolysis of cellulose derivatives. *ACS Symposium Series*, 201-216.

Saha, B. (2001) Xylanase from a newly isolated *Fusarium verticillioides* capable of utilizing corn fiber xylan. *Applied Microbiology and Biotechnology*, 56(5-6), 762-766.

Saha, B. (2002) Production, purification and properties of xylanase from a newly isolated *Fusarium proliferatum*. *Process Biochemistry*, 37(11), 1279-1284.

Saha, B. (2004) Production, purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*. *Process Biochemistry*, 39(12), 1871-1876.

Saha, B. and Bothast, R. (1999) Pretreatment and enzymatic saccharification of corn fiber. *Applied Biochemistry and Biotechnology*, 76(2), 65-78.

Saikkonen, K., Faeth, S., Helander, M. and Sullivan, T. (1998) Fungal endophytes: a continuum of interactions with host plants. *Annual Review of Ecology and Systematics*, 29(1), 319-343.

Sakai, K., Yamauchi, T., Nakasu, F. and Ohe, T. (1996) Biodegradation of cellulose acetate by *Neisseria sicca*. *Bioscience, Biotechnology, and Biochemistry*, 60(10), 1617-1622.

Samios, E., Dart, R. and Dawkins, J. (1997) Preparation, characterization and biodegradation studies on cellulose acetates with varying degrees of substitution. *Polymer*, 38, 3045-3054.

Sapkota, A., Berger, S. and Vogel, T. (2010) Human pathogens abundant in the bacterial metagenome of cigarettes. *Environmental Health Perspectives*, 118(3), 351-356.

Sarkar, N., Ghosh, S., Bannerjee, S. and Aikat, K. (2012) Bioethanol production from agricultural wastes: An overview. *Renewable Energy*, 37, 19-27.

Schmidt, S., Sunyaev, S., Bork, P. and Dandekar, T. (2003) Metabolites: a helping hand for pathway evolution? *Trends in Biochemical Sciences*, 28(6), 336-341.

Schoch, C., Seifert, K., Huhndorf, S., Robert, V., Spouge, J., Levesque, C., Chen, W., Bolchacova, E., Voigt, K., Crous, P., Miller, A., Wingfield, M., Aime, M., An, K., Bai, F., Barreto, R., Begerow, D., Bergeron, M., Blackwell, M., Boekhout, T., Bogale, M., Boonyuen, N., Burgaz, A., Buyck, B., Cai, L., Cai, Q., Cardinali, G., Chaverri, P., Coppins, B., Crespo, A., Cubas, P., Cummings, C., Damm, U., de Beer, Z., de Hoog, G.,

Del-Prado, R., Dentinger, B., Dieguez-Uribeondo, J., Divakar, P., Douglas, B., Duenas, M., Duong, T., Eberhardt, U., Edwards, J., Elshahed, M., Fliegerova, K., Furtado, M., Garcia, M., Ge, Z., Griffith, G., Griffiths, K., Groenewald, J., Groenewald, M., Grube, M., Gryzenhout, M., Guo, L., Hagen, F., Hambleton, S., Hamelin, R., Hansen, K., Harrold, P., Heller, G., Herrera, C., Hirayama, K., Hirooka, Y., Ho, H., Hoffmann, K., Hofstetter, V., Hognabba, F., Hollingsworth, P., Hong, S., Hosaka, K., Houbraken, J., Hughes, K., Huhtinen, S., Hyde, K., James, T., Johnson, E., Johnson, J., Johnston, P., Jones, E., Kelly, L., Kirk, P., Knapp, D., Koljalg, U., Kovacs, G., Kurtzman, C., Landvik, S., Leavitt, S., Liggenstoffer, A., Liimatainen, K., Lombard, L., Luangsa-ard, J., Lumbsch, H., Maganti, H., Maharachchikumbura, S., Martin, M., May, T., McTaggart, A., Methven, A., Meyer, W., Moncalvo, J., Mongkolsamrit, S., Nagy, L., Nilsson, R., Niskanen, T., Nyilasi, I., Okada, G., Okane, I., Olariaga, I., Otte, J., Papp, T., Park, D., Petkovits, T., Pino-Bodas, R., Quaedvlieg, W., Raja, H., Redecker, D., Rintoul, T., Ruibal, C., Sarmiento-Ramirez, J., Schmitt, I., Schussler, A., Shearer, C., Sotome, K., Stefani, F., Stenroos, S., Stielow, B., Stockinger, H., Suetrong, S., Suh, S., Sung, G., Suzuki, M., Tanaka, K., Tedersoo, L., Telleria, M., Tretter, E., Untereiner, W., Urbina, H., Vagvolgyi, C., Vialle, A., Vu, T., Walther, G., Wang, Q., Wang, Y., Weir, B., Weiss, M., White, M., Xu, J., Yahr, R., Yang, Z., Yurkov, A., Zamora, J., Zhang, N., Zhuang, W. and Schindel, D. (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences*, 109(16), 6241-6246.

Schoch, C., Sung, G., López-Giráldez, F., Townsend, J., Miadlikowska, J., Hofstetter, V., Robbertse, B., Matheny, P., Kauff, F., Wang, Z., Gueidan, C., Andrie, R., Trippe, K., Ciufetti, L., Wynns, A., Fraker, E., Hodkinson, B., Bonito, G., Groenewald, J., Arzanlou, M., Sybren de Hoog, G., Crous, P., Hewitt, D., Pfister, D., Peterson, K., Gryzenhout, M., Wingfield, M., Aptroot, A., Suh, S., Blackwell, M., Hillis, D., Griffith, G., Castlebury, L., Rossman, A., Lumbsch, H., Lücking, R., Büdel, B., Rauhut, A., Diederich, P., Ertz, D., Geiser, D., Hosaka, K., Inderbitzin, P., Kohlmeyer, J., Volkmann-Kohlmeyer, B., Mostert, L., O'Donnell, K., Sipman, H., Rogers, J., Shoemaker, R., Sugiyama, J., Summerbell, R., Untereiner, W., Johnston, P., Stenroos, S., Zuccaro, A., Dyer, P., Crittenden, P., Cole, M., Hansen, K., Trappe, J., Yahr, R., Lutzoni, F. and Spatafora, J. (2009) The Ascomycota Tree of Life: a phylum-wide phylogeny clarifies the origin and

evolution of fundamental reproductive and ecological traits. *Systematic Biology*, 58(2), 224-239.

Schroers, H., Gräfenhan, T., Nirenberg, H. and Seifert, K. (2011) A revision of *Cyanonectria* and *Geejayessia* gen. nov., and related species with *Fusarium*-like anamorphs. *Studies in Mycology*, 68, 115-138.

Schroers, H., O'Donnell, K., Lamprecht, S., Kammeyer, P., Johnson, S., Sutton, D., Rinaldi, M., Geiser, D. and Summerbell, R. (2009) Taxonomy and phylogeny of the *Fusarium dimerum* species group. *Mycologia*, 101(1), 44-70.

Schwarz, P., Bretagne, S., Gantier, J., Garcia-Hermoso, D., Lortholary, O., Dromer, F. and Dannaoui, E. (2006) Molecular identification of Zygomycetes from culture and experimentally infected tissues. *Journal of Clinical Microbiology*, 44(2), 340-349.

Sexton, K. and Adgate, J. (1999) Looking at environmental justice from an environmental health perspective. *Journal of Exposure Science & Environmental Epidemiology*, 9(1), 3-8.

Sharma, K. (2016) Fungal genome sequencing: basic biology to biotechnology. *Critical Reviews in Biotechnology*, 36, 743-759.

Shimonaka, A., Koga, J., Baba, Y., Nishimura, T., Murashima, K., Kubota, H. and Kono, T. (2006) Specific characteristics of family 45 endoglucanases from *Mucorales* in the use of textiles and laundry. *Bioscience, Biotechnology, and Biochemistry*, 70(4), 1013-1016.

Shin, H. and Chen, R. (2006) Production and characterization of a type B feruloyl esterase from *Fusarium proliferatum* NRRL 26517. *Enzyme and Microbial Technology*, 38(4), 478-485.

Silva, E., Fialho, A., Sá-Correia, I., Burns, R. and Shaw, L. (2004) Combined bioaugmentation and biostimulation to cleanup soil contaminated with high concentrations of atrazine. *Environmental Science & Technology*, 38, 632-637.

Simon, J., Müller, H., Koch, R. and Müller, V. (1998) Thermoplastic and biodegradable polymers of cellulose. *Polymer Degradation and Stability*, 59(1-3), 107-115.

Singh, M., Srivastava, P., Verma, P., Kharwar, R., Singh, N. and Tripathi, R. (2015) Soil fungi for mycoremediation of arsenic pollution in agriculture soils. *Journal of Applied Microbiology*, 119(5), 1278-1290.

Singh, R., Kumar, M., Mittal, A. and Mehta, P. (2016) Microbial enzymes: industrial progress in 21st century. *3 Biotech*, 6(2).

Skovgaard, K. and Rosendahl, S. (1998) Comparison of intra- and extracellular isozyme banding patterns of *Fusarium oxysporum*. *Mycological Research*, 102(9), 1077-1084.

Slaughter, E., Gersberg, R., Watanabe, K., Rudolph, J., Stransky, C. and Novotny, T. (2011) Toxicity of cigarette butts, and their chemical components, to marine and freshwater fish. *Tobacco Control*, 20, 25-29.

Spatafora, J., Aime, C., Grigoriev, I., Martin, F., Stajich, J. and Blackwell, M. (2017) The Fungal Tree of Life: from molecular systematics to genome-scale phylogenies. *Microbiology Spectrum*, 5, 5.

Spezio, M., Wilson, D. and Karplus, P. (1993) Crystal structure of the catalytic domain of a thermophilic endocellulase. *Biochemistry*, 32(38), 9906-9916.

Srivastava, S. (2015) Bioremediation technology: a greener and sustainable approach for restoration of environmental pollution. In: Kaushik G. (eds) Applied Environmental Biotechnology: Present Scenario and Future Trends. Springer, New Delhi.

Stielow, J., Lévesque, C., Seifert, K., Meyer, W., Irinyi, L., Smits, D., Renfurm, R., Verkley, G., Groenewald, M., Chaduli, D., Lomascolo, A., Welti, S., Lesage-Meessen, L., Favel, A., Al-Hatmi, A., Damm, U., Yilmaz, N., Houbraken, J., Lombard, L., Quaedvlieg, W., Binder, M., Vaas, L., Vu, D., Yurkov, A., Begerow, D., Roehl, O., Guerreiro, M., Fonseca, A., Samerpitak, K., van Diepeningen, A., Dolatabadi, S., Moreno, L., Casaregola, S., Mallet, S., Jacques, N., Roscini, L., Egidi, E., Bizet, C., Garcia-Hermoso, D., Martín, M., Deng, S., Groenewald, J., Boekhout, T., de Beer, Z., Barnes, I., Duong, T., Wingfield, M., de Hoog, G., Crous, P., Lewis, C., Hambleton, S., Moussa, T., Al-Zahrani, H., Almaghrabi, O., Louis-Seize, G., Assabgui, R., McCormick, W., Omer, G., Dukik, K., Cardinali, G., Eberhardt, U., de Vries, M. and Robert, V. (2015) One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. *Persoonia - Molecular Phylogeny and Evolution of Fungi*, 35(1), 242-263.

Su, J., Xu, J., Lu, W. and Lin, G. (2006) Enzymatic transformation of ginsenoside Rg3 to Rh2 using newly isolated *Fusarium proliferatum* ECU2042. *Journal of Molecular Catalysis B: Enzymatic*, 38, 113-118.

Su, J., Xu, J., Yu, H., He, Y., Lu, W. and Lin, G. (2009) Properties of a novel β-glucosidase from *Fusarium proliferatum* ECU2042 that converts ginsenoside Rg3 into Rh2. *Journal of Molecular Catalysis B: Enzymatic*, 57, 278-283.

Suaria, G., Achtypi, A., Perold, V., Lee, J., Pierucci, A., Bornman, T., Aliani, S. and Ryan, P. (2020) Microfibers in oceanic surface waters: A global characterization. *Science Advances*, 6(23), 84-93.

Summerbell, R., Richardson, S. and Kane, J. (1988) *Fusarium proliferatum* as an agent of disseminated infection in an immunosuppressed patient. *Journal of Clinical Microbiology*, 26(1), 82-87.

Summerell, B. (2019) Resolving *Fusarium*: current status of the genus. *Annual Review of Phytopathology*, 57(1), 323-339.

Sun, S., Lui, Q., Han, L., Ma, Q., He, S., Li, X., Zhang, H., Zhang, J., Liu, X. and Wang, L. (2018) Identification and characterization of *Fusarium proliferatum*, a new species of fungi that cause fungal keratitis. *Scientific Reports*, 8(1), 1-9.

Szczęsna-Antczak, M., Struszczyk-Świta, K., Rzyska, M., Szeląg, J., Stańczyk, Ł. and Antczak, T. (2018) Oil accumulation and in situ trans/esterification by lipolytic fungal biomass. *Bioresource Technology*, 265, 110-118.

Szécsi, Á., Turóczi, G. and Bordás, B. (1995) Analysis of esterase zymograms of *Fusarium sambucinum* and related species. *Mycopathologia*, 129(3), 165-171.

Takano, M. and Hoshino, K. (2012) Direct ethanol production from rice straw by coculture with two high-performing fungi. *Frontiers of Chemical Science and Engineering*, 6(2), 139-145.

Taylor, E., Meriwether, B. and Park, J. (1963) The hydrolysis of p-nitrophenyl acetate catalyzed by 3-phosphoglyceraldehyde dehydrogenase crystallized from yeast. *Journal of Biological Chemistry*, 238, 734-740.

Teather, R. and Wood, P. (1982) Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Applied and Environmental Microbiology*, 43(4), 777-780.

Testa, A., Di, A., Rao. M., Monti, M., Pedata, P. and Van der Lee, T. (2012) A genomic approach for identification of fungal genes involved in pentachlorophenol degradation. *Journal of Advanced Research*, 9, 1386–1389.

Thiele, H. and Rehm, H. (1979) Formation and splitting of esters in subterminal oxidation of dodecane by *Fusarium lini*. *European Journal of Applied Microbiology and Biotechnology*, 6, 361-369.

Thrane, U. and Seifert, K. (2000) *Fusarium* Link: Fr. In Samson, R. A., Hoekstra, E. S.,Frisvad, J. C. and Filtenborg, O. (Eds.), Introduction to food- and airborne fungi. Centraalbureau Voor Schimmecultures, Utrecht, 120-157.

Tibpromma, S., Hyde, K., Jeewon, R., Maharachchikumbura, S., Liu, J., Bhat, D., Jones, E., McKenzie, E., Camporesi, E., Bulgakov, T., Doilom, M., de Azevedo Santiago, A., Das, K., Manimohan, P., Gibertoni, T., Lim, Y., Ekanayaka, A., Thongbai, B., Lee, H., Yang, J., Kirk, P., Sysouphanthong, P., Singh, S., Boonmee, S., Dong, W., Raj, K., Latha, K., Phookamsak, R., Phukhamsakda, C., Konta, S., Jayasiri, S., Norphanphoun, C., Tennakoon, D., Li, J., Dayarathne, M., Perera, R., Xiao, Y., Wanasinghe, D., Senanayake, I., Goonasekara, I., de Silva, N., Mapook, A., Jayawardena, R., Dissanayake, A., Manawasinghe, I., Chethana, K., Luo, Z., Hapuarachchi, K., Baghela, A., Soares, A., Vizzini, A., Meiras-Ottoni, A., Mešić, A., Dutta, A., de Souza, C., Richter, C., Lin, C., Chakrabarty, D., Daranagama, D., Lima, D., Chakraborty, D., Ercole, E., Wu, F., Simonini, G., Vasquez, G., da Silva, G., Plautz, H., Ariyawansa, H., Lee, H., Kušan, I., Song, J., Sun, J., Karmakar, J., Hu, K., Semwal, K., Thambugala, K., Voigt, K., Acharya, K., Rajeshkumar, K., Ryvarden, L., Jadan, M., Hosen, M., Mikšík, M., Samarakoon, M., Wijayawardene, N., Kim, N., Matočec, N., Singh, P., Tian, Q., Bhatt, R., de Oliveira, R., Tulloss, R., Aamir, S., Kaewchai, S., Marathe, S., Khan, S., Hongsanan, S., Adhikari, S., Mehmood, T., Bandyopadhyay, T., Svetasheva, T., Nguyen, T., Antonín, V., Li, W., Wang, Y., Indoliya, Y., Tkalčec, Z., Elgorban, A., Bahkali, A., Tang, A., Su, H., Zhang, H., Promputtha, I., Luangsa-ard, J., Xu, J., Yan, J., Ji-Chuan, K., Stadler, M., Mortimer, P., Chomnunti, P., Zhao, Q., Phillips, A., Nontachaiyapoom, S., Wen, T. and Karunarathna, S. (2017) Fungal diversity notes 491–

602: taxonomic and phylogenetic contributions to fungal taxa. *Fungal Diversity*, 83(1), 1-261.

Tigini, V., Prigione, V., Di Toro, S., Fava, F. and Varese, G. (2009) Isolation and characterisation of polychlorinated biphenyl (PCB) degrading fungi from a historically contaminated soil. *Microbial Cell Factories*, 8(1), 5.

Tkacz, J. and Lange, L. (2004) *Advances In Fungal Biotechnology For Industry, Agriculture And Medicine*. New York: Kluwer, 3 - 15.

Tobacco Atlas. [online] Available at: <https://tobaccoatlas.org> [Accessed 28 August 2020].

Torkashvand, J. and Farzadkia, M. (2019) A systematic review on cigarette butt management as a hazardous waste and prevalent litter: control and recycling. *Environmental Science and Pollution Research*, 26, 11618-11630.

Torkashvand, J., Farzadkia, M., Sobhi, H. and Esrafili, A. (2020) Littered cigarette butt as a well-known hazardous waste: a comprehensive systematic review. *Journal of Hazardous Materials*, 383, 121242.

Tortorano, A., Prigitano, A., Esposto, M., Arsic Arsenijevic, V., Kolarovic, J., Ivanovic, D., Paripovic, L., Klingspor, L., Nordøy, I., Hamal, P., Arikan Akdagli, S., Ossi, C., Grancini, A., Cavanna, C., Lo Cascio, G., Scarparo, C., Candoni, A., Caira, M. and Drogari Apiranthitou, M. (2014) European Confederation of Medical Mycology (ECMM) epidemiological survey on invasive infections due to *Fusarium* species in Europe. *European Journal of Clinical Microbiology & Infectious Diseases*, 33(9), 1623-1630.

Tyagi, M., da Fonseca, M. and de Carvalho, C. (2010) Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. *Biodegradation*, 22, 231-241.

Ueno, A., Ito, Y., Yumoto, I. and Okuyama, H. (2007) Isolation and characterization of bacteria from soil contaminated with diesel oil and the possible use of these in autochthonous bioaugmentation. *World Journal of Microbiology and Biotechnology*, 23, 1739-1745.

Urgun-Demirtas, M., Stark, B. and Pagilla, K. (2008) Use of genetically engineered micro-organisms (GEMs) for the bioremediation of contaminants. *Critical Reviews in Biotechnology*, 26, 145-164.

Valášková, V. and Baldrian, P. (2006) Degradation of cellulose and hemicelluloses by the brown rot fungus *Piptoporus betulinus* – production of extracellular enzymes and characterization of the major cellulases. *Microbiology*, 152, 3613-3622.

Varadarajan, H. and Shikha, S. (2014) Biodiversity characterization of bacterial and fungal isolates from gold electroplating industry effluents. *Journal of Applied & Environmental Microbiology*, 2(5), 212-219.

Veena, V. (2011) Isolation and characterization of β-glucosidase producing bacteria from different sources. *African Journal of Biotechnology*, 10(66), 14907-14912.

Venkatesagowda, B., Ponugupaty, E., Barbosa, A. and Dekker, R. (2012) Diversity of plant oil seed-associated fungi isolated from seven oil-bearing seeds and their potential for the production of lipolytic enzymes. *World Journal of Microbiology and Biotechnology*, 28, 71-80.

Ventorino, V., Pascale, A., Adamo, P., Rocco, C., Fiorentino, N., Mori, M., Faraco, V., Pepe, O. and Fagnano, M. (2018) Comparative assessment of autochthonous bacterial and fungal communities and microbial biomarkers of polluted agricultural soils of the Terra dei Fuochi. *Scientific Reports*, 8(1).

Vicente, G., Bautista, L., Rodríguez, R., Gutiérrez, F., Sádaba, I., Ruiz-Vázquez, R., Torres-Martínez, S. and Garre, V. (2009) Biodiesel production from biomass of an oleaginous fungus. *Biochemical Engineering Journal*, 48(1), 22-27.

Vilgalys, R. (2003) Taxonomic misidentification in public DNA databases. *New Phytologist*, 160(1), 4-5.

Villemur, R., Lacasse, M. and Morin, A. (2009) Monitoring the bacterial and fungal biota of eleven tobacco grades stored at three different locations. *Beiträge zur Tabakforschung International/Contributions to Tobacco Research*, 23(6), 368-376.

Vitale, R., de Hoog, G., Schwarz, P., Dannaoui, E., Deng, S., Machouart, M., Voigt, K., van de Sande, W., Dolatabadi, S., Meis, J. and Walther, G. (2011) Antifungal susceptibility

and phylogeny of opportunistic members of the order *Mucorales*. *Journal of Clinical Microbiology*, 50(1), 66-75.

Vohra, M., Manwar, J., Manmode, R., Padgilwar, S. and Patil, S. (2014) Bioethanol production: feedstock and current technologies. *Journal of Environmental Chemical Engineering*, 2(1), 573-584.

Voigt, C., Schäfer, W. and Salomon, S. (2005) A secreted lipase of *Fusarium graminearum* is a virulence factor required for infection of cereals. *The Plant Journal*, 42, 364-375.

Voigt, K., Wolf, T., Ochsenreiter, K., Nagy, G., Kaerger, K., Shelest, E. and Papp, T. (2016) 15 genetic and metabolic aspects of primary and secondary metabolism of the Zygomycetes. *Biochemistry and Molecular Biology*, 361-385.

Vujanovic, V., Hamel, C., Yergeau, E. and St-Arnaud, M. (2006) Biodiversity and biogeography of *Fusarium* species from northeastern North American asparagus fields based on microbiological and molecular approaches. *Microbial Ecology*, 51(2), 242-255.

Waalwijk, C., de Koning, J., Baayen, R. and Gams, W. (1996) Discordant groupings of *Fusarium* spp. from sections Elegans, Liseola and Dlaminia based on ribosomal ITS1 and ITS2 sequences. *Mycologia*, 88(3), 361.

Wagner, A. (2011) The molecular origins of evolutionary innovations. *Trends in Genetics*, 27(10), 397-410.

Wagner, L., Stielow, J., de Hoog, G., Bensch, K., Schwartze, V., Voigt, K., Alastruey-Izquierdo, A., Kurzai, O. and Walther, G. (2019) A new species concept for the clinically relevant *Mucor circinelloides* complex. *Persoonia - Molecular Phylogeny and Evolution of Fungi*, 44, 67-97.

Waksman, S. (1922) A method for counting the number of fungi in the soil. *Journal of Bacteriology*, 7(3), 339-341.

Walther, G., Pawłowska, J., Alastruey-Izquierdo, A., Wrzosek, M., Rodriguez-Tudela, J., Dolatabadi, S., Chakrabarti, A. and de Hoog, G. (2013) DNA barcoding in *Mucorales*: an inventory of biodiversity. *Persoonia - Molecular Phylogeny and Evolution of Fungi*, 30(1), 11-47.

Walther, G., Wagner, L. and Kurzai, O. (2019) Updates on the taxonomy of *Mucorales* with an emphasis on clinically important taxa. *Journal of Fungi*, 5(4), 106.

Walton, J. (1994) Deconstructing the cell wall. *Plant Physiology*, 104(4), 1113-1118.

Wanjari, S., Prabhu, C., Satyanarayana, T., Vinu, A. and Rayalu, S. (2012) Immobilization of carbonic anhydrase on mesoporous aluminosilicate for carbonation reaction. *Microporous and Mesoporous Materials*, 160, 151-158.

Weber, W. and Corseuil, H. (1994) Inoculation of contaminated subsurface soils with enriched indigenous microbes to enhance bioremediation rates. *Water Research*, 28, 1407-1414.

Wei, X., Deng, X., Cai, D., Ji, Z., Wang, C., Yu, J., Li, J. and Chen, S. (2014) Decreased tobacco-specific nitrosamines by microbial treatment with *Bacillus amyloliquefaciens* DA9 during the air-curing process of burley tobacco. *Journal of Agricultural and Food Chemistry*, 62(52), 12701-12706.

Welty, R. (1972) Fungi isolated from flue-cured tobacco sold in southeast United States, 1968-1970. *Applied Microbiology*, 24(3), 518-520.

White, M. and Garcin, E. (2017) D-Glyceraldehyde-3-phosphate dehydrogenase structure and function. *Subcellular Biochemistry*, 413-453.

White, T., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: a guide to methods and applications. Academic Press, New York, USA: 315–322.

Wiemann, P., Sieber, C., von Bargen, K., Studt, L., Niehaus, E., Espino, J., Huß, K., Michielse, C., Albermann, S., Wagner, D., Bergner, S., Connolly, L., Fischer, A., Reuter, G., Kleigrewe, K., Bald, T., Wingfield, B., Ophir, R., Freeman, S., Hippler, M., Smith, K., Brown, D., Proctor, R., Münsterkötter, M., Freitag, M., Humpf, H., Güldener, U. and Tudzynski, B. (2013) Deciphering the cryptic genome: genome-wide analyses of the rice pathogen *Fusarium fujikuroi* reveal complex regulation of secondary metabolism and novel metabolites. *PLoS Pathogens*, 9(6), e1003475.

Wingfield, M., Hammerbacher, A., Ganley, R., Steenkamp, E., Gordon, T., Wingfield, B. and Coutinho, T. (2008) Pitch canker caused by *Fusarium circinatum*– a growing threat to pine plantations and forests worldwide. *Australasian Plant Pathology*, 37(4), 319.

Woodall, L., Sanchez-Vidal, A., Canals, M., Paterson, G., Coppock, R., Sleight, V., Calafat, A., Rogers, A., Narayanaswamy, B. and Thompson, R. (2014) The deep sea is a major sink for microplastic debris. *Royal Society Open Science*, 1, 140317.

World Health Organization (2017). Tobacco and Its Environmental Impact: An Overview.

World Health Organization (2019). WHO report on the Global Tobacco Epidemic, pp.1-35.

Wright, S., Rowe, D., Reid, M., Thomas, K. and Galloway, T. (2015) Bioaccumulation and biological effects of cigarette litter in marine worms. *Scientific Reports*, 5(1), 14119.

Wu, B., Hussain, M., Zhang, W., Stadler, M., Liu, X. and Xiang, M. (2019) Current insights into fungal species diversity and perspective on naming the environmental DNA sequences of fungi. *Mycology*, 10(3), 127-140.

Xie, Y., Gao, Y. and Chen, Z. (2004) Purification and characterization of an extracellular β-glucosidase with high transglucosylation activity and stability from *Aspergillus niger* No. 5.1. *Applied Biochemistry and Biotechnology*, 119, 229-240.

Xiros, C., Katapodis, P. and Christakopoulos, P. (2011) Factors affecting cellulose and hemicellulose hydrolysis of alkali treated brewers spent grain by *Fusarium oxysporum* enzyme extract. *Bioresource Technology*, 102(2), 1688-1696.

Xiros, C., Topakas, E., Katapodis, P. and Christakopoulos, P. (2008) Evaluation of *Fusarium oxysporum* as an enzyme factory for the hydrolysis of brewer's spent grain with improved biodegradability for ethanol production. *Industrial Crops and Products*, 28(2), 213-224.

Xu, J., Wang, X., Hu, L., Xia, J., Wu, Z., Xu, N., Dai, B. and Wu, B. (2015) A novel ionic liquid-tolerant *Fusarium oxysporum* BN secreting ionic liquid-stable cellulase: consolidated bioprocessing of pretreated lignocellulose containing residual ionic liquid. *Bioresource Technology*, 181, 18-25.

Yakop, F., Taha, H. and Shivanand, P. (2019) Isolation of fungi from various habitats and their possible bioremediation. *Current Science*, 116(5), 733.

Yang, B., Dai, Z., Ding, S. and Wyman, C. (2011) Enzymatic hydrolysis of cellulosic biomass. *Biofuels*, 2(4), 421-449.

Yang, J., Duan, Y., Chen, C., Li, Q., Huang, J. and Zhang, K. (2008). Identification and phylogenesis analysis of cultivable microorganisms on tobacco leaf surface during aging. *Tobacco Science & Technology*, 11, 51–55.

Yi, Y., Wang, Z., Zhang, K., Yu, G. and Duan, X. (2008) Sediment pollution and its effect on fish through food chain in the Yangtze River. *International Journal of Sediment Research*, 23(4), 338-347.

Zeller, K., Summerell, B., Bullock, S. and Leslie, J. (2003) *Gibberella konza* (F*usarium konzum*) sp. nov. from prairie grasses, a new species in the *Gibberella fujikuroi* species complex. *Mycologia*, 95(5), 943.

Zhang, X., Yang, H. and Cui, Z. (2017) *Mucor circinelloides*: efficiency of bioremediation response to heavy metal pollution. *Toxicology Research*, 6(4), 442-447.

Zhang, Z., Zhang, G., Chen, J., Wei, B., Lan, B. and Du, J. (2018) Diversity of culturable fungi on surface of tobacco leaves during aging. *Tobacco Science & Technology*, 51(12), 23–30.

Zhao, Z., Ramachandran, P., Kim, T., Chen, Z., Jeya, M. and Lee, J. (2013) Characterization of an acid-tolerant β-1,4-glucosidase from *Fusarium oxysporum* and its potential as an animal feed additive. *Applied Microbiology and Biotechnology*, 97, 10003-10011.

Zhou, J., Yu, L., Zhang, J., Zhang, X., Xue, Y., Liu, J. and Zou, X. (2020) Characterization of the core microbiome in tobacco leaves during aging. *Microbiology Open*, 9(3).

Supplementary data

Table 4: Summary of chemicals identified in cigarette emissions and extractions (Adapted from Poppendieck *et al*., 2016).

Chemical	CAS	MW (g/mol)	Component	Source	Reference
Alcohols					
Ethanol	64-17-5	46.1	Tipping paper	Air	Huang *et al*., 2014
2-furfuryl Alcohol	98-00-0	98.1	Cigarette filter	Air	Fukuhara *et al*., 1985
Isopropanol	67-63-0	60.1	Tipping paper	Air	Huang *et al*., 2014
Methanol	67—56-1	32.0	Cigarette filter	Air	You *et al*., 2014
1-methoxy-2-propanol	107-98-2	90.1	Tipping paper	Air	Huang *et al*., 2014
1-ethoxy-2-propanol	1569-02-4	104.2	Tipping paper	Air	Huang *et al*., 2014
Alkaloids					
7-Carbaldehyde camptothecin	80758-83-4	376.4	Cigarette filter	Water	Zhao *et al*., 2010
Aromatic amines					
Nicotine	22083-74-5	162.2	Cigarette filter	Water	Zhao *et al*., 2010; Green *et al*., 2014
Hexaconazole	79983-74-4	314.2	Cigarette filter	Water	Zhao *et al*., 2010

Imidocarb	27885-92-3	348.4	Cigarette filter	Water	Zhao *et al.*, 2010
Cotinine	486-56-6	176.2	Cigarette filter	Water	Zhao *et al.*, 2010
2-(Pyridin-3-yl) Pyrrolidine-1-Carbaldehyde	3000-81-5	176.2	Cigarette filter	Water	Zhao *et al.*, 2010
Sulfadoxine	2447-57-6	310.3	Cigarette filter	Water	Zhao *et al.*, 2010
Carbonyls					
Acetol	116-09-6	74.1	Cigarette filter	Air	Fukuhara *et al.*, 1985
Acetone	67-64-1	58.1	Tipping paper	Air	Huang *et al.*, 2014
Acetone	67-64-1	58.1	Cigarette filter	Extract solution	Yu *et al.*, 2013
Butyl acetate	123-86-4	116.2	Tipping paper	Air	Huang *et al.*, 2014
Cyclohexanone	108-94-1	98.1	Tipping paper	Air	Huang *et al.*, 2014
2-Pentanone	107-87-9	86.1	Cigarette filter	Air	Fukuhara *et al.*, 1985
n-propanol	71-23-8	60.1	Tipping paper	Air	Huang *et al.*, 2014
n-propyl acetate	109-60-4	102.1	Tipping paper	Air	Huang *et al.*, 2014
Cyclopentanone	120-92-3	84.1	Cigarette filter	Air	Fukuhara *et al.*, 1985
2-Cycopentenone	930-30-3	82.1	Cigarette filter	Air	Fukuhara *et al.*, 1985

2-Methylcyclo pentenone	1120-73-6	96.1	Cigarette filter	Air	Fukuhara *et al.*, 1985
4-methyl-2-pentanone	108-10-1	100.2	Tipping paper	Air	Huang *et al.*, 2014
Methyl n-Butyl Ketone	591-78-6	100.2	Tipping paper	Air	Huang *et al.*, 2014
n-butyl alcohol	71-36-3	74.1	Tipping paper	Air	Huang *et al.*, 2014
Ethyl acetate	141-78-6	88.1	Tipping paper	Air	Huang *et al.*, 2014
3-Furaldehyde	498-60-2	96.1	Cigarette filter	Air	Fukuhara *et al.*, 1985
2-Furaldehyde	98-01-1	96.1	Cigarette filter	Air	Fukuhara *et al.*, 1985
Formaldehyde	50-00-0	30.0	Cigarette filter	Extract solution	Yu *et al.*, 2013
Isopropyl acetate	108-21-4	102.1	Tipping paper	Air	Huang *et al.*, 2014
Acetaldehyde	75-07-0	44.1	Cigarette filter	Extract solution	Yu *et al.*, 2013
Acrolein	107-02-8	56.1	Cigarette filter	Extract solution	Yu *et al.*, 2013
Propionaldehyde	123-38-6	58.1	Cigarette filter	Extract solution	Yu *et al.*, 2013
Protoanemonin	108-28-1	96.08	Cigarette filter	Air	Fukuhara *et al.*, 1985
Crotonaldehyde	4170-30-3	70.09	Cigarette filter	Extract solution	Yu *et al.*, 2013
2-Butanone	78-93-3	72.1	Cigarette filter	Extract solution	Yu *et al.*, 2013
Butyraldehyde	123-72-8	72.1	Cigarette filter	Extract solution	Yu *et al.*, 2013

Acetol Formate	116-09-6	74.1	Cigarette filter	Air	Fukuhara *et al*., 1985
3-Methyl-2-Cyclopentenone	2758-18-1	96.1	Cigarette filter	Air	Fukuhara *et al*., 1985
2,3-Pentanedione	600-14-6	100.1	Cigarette filter	Air	Fukuhara *et al*., 1985
Hydrocarbons					
Ethyl Benzene	100-41-4	106.2	Cigarette filter	Air	Fukuhara *et al*., 1985; Ji *et al*., 2015
Ethyl Benzene	100-41-4	106.2	Tipping paper	Air	Huang *et al*., 2014
Benzene	71-43-2	78.1	Tipping paper	Air	Huang *et al*., 2014
Benzene	71-43-2	78.1	Cigarette filter	Air	Ji *et al*., 2015
3,3-Dimethyl-1-Butene	558-37-2	84.2	Cigarette filter	Air	Fukuhara *et al*., 1985
Pyridine	110-86-1	79.1	Cigarette filter	Air	Fukuhara *et al*., 1985
Cyanobenzene	100-47-0	103.1	Cigarette filter	Air	Fukuhara *et al*., 1985
Cyclooctatetraene	629-20-9	104.2	Cigarette filter	Air	Fukuhara *et al*., 1985
Isocapronitrile	542-54-1	97.2	Cigarette filter	Air	Fukuhara *et al*., 1985

Toluene	108-88-3	92.1	Cigarette Filter	Air	Fukuhara *et al*., 1985; Ji *et al*., 2014
Toluene	108-88-3	92.1	Tipping paper	Air	Huang *et al*., 1985
p-Xylene	106-42-3	106.2	Cigarette filter	Air	Ji *et al*., 2015
m-Xylene	108-38-3	106.2	Cigarette filter	Air	Ji *et al*., 2015
m-Xylene	108-.8-3	106.2	Tipping paper	Air	Huang *et al*., 2014
o-Xylene	95-47-6	106.2	Tipping paper	Air	Huang *et al*., 2014
o-Xylene	95-47-6	106.2	Cigarette filter	Air	Ji *et al*., 2015
Styrene	100-42-5	104.2	Tipping paper	Air	Huang *et al*., 2014
Styrene	100-42-5	104.2	Cigarette filter	Air	Ji *et al*., 2015
Xylene	1330-20-7	106.2	Cigarette filter	Air	Fukuhara *et al*., 1985
Insecticides					
Maleic Hydrazide	123-33-1	112.1	Cigarette filter	Extract solution	Zhang *et al*., 2012
Chlorantraniliprole	500008-45-7	483.2	Cigarette filter	Extract solution	Goddamidi *et al*., 2011
Imidacloprid	138261-41-3	255.7	Cigarette filter	Extract solution	Clark *et al*., 1998
Metals					

Aluminum	7429-90-5	27.0	Cigarette filter	Water	Moerman and Potts, 2011
Antiminy	7440-36-0	121.8	Cigarette filter	Cigarette filter	Wu *et al.*, 1997
Arsenic	7440-38-2	74.9	Cigarette filter	Cigarette filter	Wu *et al.*, 1997
Barium	7440-39-3	137.3	Cigarette filter	Water	Moerman and Potts, 2011
Cadmium	7440-43-9	112.4	Cigarette filter	Water	Moerman and Potts, 2011
Cadmium	7440-43-9	112.4	Cigarette filter	Extract solution	Pelit *et al.*, 2013
Cadmium	7440-43-9	112.4	Cigarette filter	Cigarette filter	Wu *et al.*, 1997
Cadmium	7440-43-9	112.4	Cigarette filter	Extract solution	Galazyn-Sidorczuk *et al.*, 2011
Cadmium	7440-43-9	112.4	Unburned tobacco	Extract solution	Wand and Finlayson-Pitts, 2003
Chromium	7440-47-3	52.0	Cigarette filter	Extract solution	Wang and Finlayson-Pitts, 2003
Cobalt	7440-48-4	58.9	Cigarette filter	Cigarette filter	Wu *et al.*, 1997
Copper	7440-50-8	63.6	Cigarette filter	Extract solution	Pelit *et al.*, 2013
Mercury	7439-97-6	200.6	Cigarette filter	Extract solution	Wang *et al.*, 2007

Mercury	7439-97-6	200.6	Cigarette filter	Cigaretter filter	Kowalski and Wiercinski, 2009
Iron	7439-89-6	55.9	Cigarette filter	Extract solution	Wang and Finlayson-Pitts, 2003
Lead	7439-92-1	207.2	Cigarette filter	Extract solution	Galazyn-Sidorczuk *et al*., 2008; Huang *et al*., 2013; Wu *et al*., 2012
Lead	7439-92-1	207.2	Cigarette filter	Water	Moerman and Potts, 2011
Manganese	7439-96-5	54.9	Cigarette filter	Water	Moerman and Potts, 2011
Manganese	7439-96-5	54.9	Cigarette filter	Extract solution	Pelit *et al*., 2013
Nickel	7440-02-0	58.7	Cigarette filter	Water	Moerman and Potts, 2011
Potassium	7440-09-7	39.1	Cigarette filter	Cigarette filter	Wu *et al*., 1997
Strontium	7440-24-6	87.6	Cigarette filter	Water	Moerman and Potts, 2011
Titanium	7440-32-6	47.9	Cigarette filter	Water	Moerman and Potts, 2011

Zinc	7440-66-6	65.4	Cigarette filter	Extract solution	Wang and Finlayson-Pitts, 2003; Pelit *et al*., 2013
Zinc	7440-66-6	65.4	Cigarette filter	Cigarette filter	Wu *et al*., 1997
Nitroasmines					
N-Nitrosonornicotine	16543-55-8	177.0	Cigarette filter	Water	Zhoa *et al*., 2010
NPAHs and PAHs					
1,3-dinitronaphthalene			Cigarette filter	Extract solution	Verdolotti *et al*., 2012
9-nitroanthracene			Cigarette filter	Extract solution	John Dane *et al*., 2002
Nitrobenzene			Cigarette filter	Extract solution	John Dane *et al*., 2002
Anthracene	120-12-7	178.2	Cigarette filter	Extract solution	Demirci, 2014
Benzo[a]pyrene	50-32-8	252.3	Cigarette filter	Water	Zhao *et al*., 2010
Benzo[a]pyrene	50-32-8	252.3	Cigarette filter	Extract solution	Ding *et al*., 2014; Zhang *et al*., 2014; Dermirci, 2014
Benz[a]anthracene	56-55-3	228.3	Cigarette filter	Extract solution	Dermirci, 2014

Benzo[b]fluoranthene	205-99-2	252.3	Cigarette filter	Extract solution	Dermirci, 2014
Fluoranthene	206-44-0	202.3	Cigarette filter	Extract solution	Dermirci, 2014
Pyrene	129-00-0	202.3	Cigarette filter	Extract solution	Dermirci, 2014
Phenols					
Catechol	120-80-9	110.1	Cigarette filter	Extract solution	Dermirci, 2014
Hydroquinone	123-31-9	110.1	Cigarette filter	Extract solution	Hu *et al.*, 2015
m-cresol	108-39-4	108.1	Cigarette filter	Extract solution	Hu *et al.*, 2015
o-cresol	95-48-7	108.1	Cigarette filter	Extract solution	Hu *et al.*, 2015
p-cresol	106-44-5	108.1	Cigarette filter	Extract solution	Hu *et al.*, 2015
Phenol	108-39-4	108.1	Cigarette filter	Extract solution	Hu *et al.*, 2015
Resorcinol	108-95-2	94.1	Cigarette filter	Extract solution	Hu *et al.*, 2015
Rutin	153-18-4	610.5	Cigarette filter	Water	Zhao *et al.*, 2010
Rutin	163-18-4	610.5	Cigarette filter	Extract solution	Sun *et al.*, 2012
2,2-Dimethyl-2,3-Dihydrobenzofuran-7-ol	1563-38-8	164.2	Cigarette filter	Water	Zhao *et al.*, 2010
1,5-Dihydroxy-Anthraquinon	117-12-4	240.2	Cigarette filter	Water	Zhao *et al.*, 2010

Phthalates					
Dibutyl Phthalate	84-74-2	278.3	Cigarette filter	Extract solution	Zhang *et al.*, 2012
Diisobutyl Phthalate	84-69-5	278.3	Cigarette filter	Extract solution	Zhang *et al.*, 2012
Di(2-ethylhexyl) phthalate	117-81-7	390.6	Cigarette filter	Extract solution	Zhang *et al.*, 2012
Pyrazines					
Pyrazine	290-37-9	80.1	Cigarette filter	Air	Fukuhara *et al.*, 1985
2-Methylpyrazine	109-08-0	94.1	Cigarette filter	Air	Fukuhara *et al.*, 1985
Pyrroles					
N-Methylpyrrole	96-54-8	81.1	Cigarette filter	Air	Fukuhara *et al.*, 1985
Pyrrole	109-97-7	67.1	Cigarette filter	Air	Fukuhara *et al.*, 1985
3-Methylpyrrole	616-43-3	81.1	Cigarette filter	Air	Fukuhara *et al.*, 1985
2-Methylpyrrole	636-41-9	81.1	Cigarette filter	Air	Fukuhara *et al.*, 1985
Terpenes					
Beta-Carotene-4,4'-Dione	472-61-7	596.8	Cigarette filter	Water	Zhao *et al.*, 2010

Xanthophylls	127-40-2	568.9	Cigarette filter	Water	Zhao *et al.*, 2010
Limonene	5989-27-5	136.2	Cigarette filter	Air	Fukuhara *et al.*, 1985
Terpenoids					
Solanesol	13190-97-1	631.1	Cigarette filter	Extract solution	Ashley *et al.*, 2010; Brinkman *et al.*, 2012; Polzin *et al.*, 2009; Watson *et al.*, 2004